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Role of anaesthetics propofol and sevoflurane on canine mammary tumour cell proliferation and neuroepithelial transforming gene 1 expression

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DOCTORAL DISSERTATION

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To my family

CONTENTS

ABSTRACT

The impact of perioperative factors on metastatic spread subsequent to surgery for primary cancer removal is of rising interest. Several studies show that anaesthesia for primary cancer surgery might impact cancer recurrence by influencing cell proliferation rate and regulating the expression of specific oncogenes like neuroepithelial transforming gene 1 (NET1), which has been associated with malignant behaviours and represents a novel prognostic marker in human epithelial cancers.

This project investigates the effects of different concentrations of clinically available propofol and sevoflurane formulations on canine mammary tumour cell proliferation and on the expression of the NET1 gene in three prospective controlled *in vitro* trials.

Primary (CIPp) and metastatic (CIPm) canine tubular adenocarcinoma cell lines were incubated with sevoflurane (1, 2.5 or 4 mM), propofol (1, 5 or 10 μg mL^{-1}) or cell culture medium (control). Proliferation was assessed after 6 hours of sevoflurane and 6 - 12 hours of propofol exposures, while NET1 expression was evaluated after 6 hours of sevoflurane and 6, 12, 24 and 48 hours of propofol exposures.

After 6 hours of propofol exposure, cell proliferation significantly increased in CIPp, while after 12 hours cell proliferation significantly increased in CIPp and decreased in CIPm.

After 6 hours of exposure, propofol significantly augmented NET1 expression in CIPp, whereas in CIPm the highest propofol concentration

significantly reduced gene expression. Propofol significantly decreased gene expression after 12 and 24 hours of exposure. No significant differences were found in CIPm after 48 hours, while the highest concentration of propofol significantly increased gene expression in CIPp after 48 hours.

Sevoflurane significantly increased cell proliferation in CIPp, while it significantly decreased cell proliferation in CIPm. The NET1 gene expression was significantly increased in CIPm after exposure to the highest sevoflurane concentration.

In conclusion, exposure to both propofol and sevoflurane mainly augmented proliferation in primary cells and diminished proliferation in metastatic ones. Conversely, propofol mostly decreased NET1 expression, while sevoflurane increased NET1 expression in metastatic cells exposed to high concentrations.

A profound comprehension of the molecular mechanisms underlying cancer spread is necessary, however, the much more variable environment in the clinical setting makes direct translation from *in vitro* to *in vivo* conditions difficult. Even though the lack of similar trials in veterinary medicine limits the chances for comprehensive comparisons, some promising results were observed in our study. Future projects assessing the influence of anaesthetic techniques on cancer recurrence are warranted.

LIST OF PUBLICATIONS

This thesis is based on the following original publications:

I Argano M, De Maria R, Rodlsberger K, Buracco P, Larenza Menzies MP (2019a) Use of a colorimetric assay (MTT) to evaluate the proliferation of canine mammary tumor cells exposed to propofol. *Canadian Journal of Veterinary Research* 83, 149-153.

II Argano M, De Maria R, Rodlsberger K, Buracco P, Larenza Menzies MP (2017) Effects of two concentrations of a clinical propofol formulation on canine mammary tumor cells NET1 gene expression: a preliminary evaluation of possible anti-metastatic properties. *Archives on Veterinary Science and Technology* AVST-136. DOI:10.29011/AVST- 136/100036.

III Argano M, De Maria R, Vogl C, Rodlsberger K, Buracco P, Larenza Menzies MP (2019b) Canine mammary tumour cells exposure to sevoflurane: effects on proliferation and neuroepithelial transforming gene 1 expression. *Veterinary Anaesthesia and Analgesia* 46(3), 369-374. DOI: 10.1016/j.vaa.2018.12.006.

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ABBREVIATIONS

ANOVA	ANalysis Of VAriance
ATP	Adenosine triphosphate
B	Baseline
C	Control
cDNA	Complementary DNA
CIPm	Metastatic tubular adenocarcinoma cell line
CIPp	Primary tubular adenocarcinoma cell line
COX	Cyclooxygenase
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphate hydrolase enzyme
IFN-γ	Interferon gamma
IL	Interleukin
LE propofol	Propofol formulation containing a lipid-based emulsion
LPA	Lysophosphatidic acid

MET	Mesenchymal-to-epithelial transition
MDA-MB-231	M.D. Anderson-Metastasis Breast-231
miR	Micro RNA
MCF-7	Michigan Cancer Foundation-7
mRNA	Messenger ribonucleic acid
MTT	(3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cell proliferation assay
NET1	Neuroepithelial transforming gene 1
NK	Natural killer
P1	LE propofol 1 μg mL^{-1}
P10	LE propofol 10 μg mL^{-1}
P5	LE propofol 5 μg mL^{-1}
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RhoA	Ras homologue gene family, member A
RNA	Ribonucleic acid
RPM	Revolutions per minute
S1	Sevoflurane 1 mM
S2.5	Sevoflurane 2.5 mM
S4	Sevoflurane 4 mM
Smad	Small mother against decapentaplegic
TGF-β	Transforming growth factor beta
TNM	Tumour size, lymph node, metastasis
ΔCt	Delta cycle threshold

1. INTRODUCTION

Malignant mammary tumours are a significant cause of morbidity and mortality in dogs. They account for 50 to 70% of cancers affecting female dogs and represent one of the most common reason for cancer-related death in this population (Vail & MacEwen 2000; Misdorp 2002; Davidson 2003; Sorenmo 2003; Karayannopoulou & Lafioniatis 2016). Comparably, breast cancer represents the most widely diffuse type of cancer in women (Porter 2009; Ferlay et al. 2014; Ghoncheh et al. 2016). In both human and canine patients, death itself is commonly caused by recurrence and metastasis (Schneider et al. 1969; Falkson et al. 1995; Vascellari et al. 2016), with tumour cells spreading via the lymphatic or cardiovascular systems towards regional lymph nodes, lungs, liver and bones (Kitchell & Loar 1997; Misdorp 2002; Sorenmo et al. 2003).

Surgical removal of the primary tumour is considered to be the most effective treatment for both human and canine patients diagnosed with malignant mammary tumours (Singletary et al. 2003; Sorenmo 2003; Chia et al. 2005; Karayannopoulou & Lafioniatis 2016). Even though other treatments, *e.g.* chemotherapy, hormonal therapy and biological therapy, are available (Hortobagyi 1998; Bernard-Marty et al. 2004), they are often less efficacious than surgical intervention (Rapiti et al. 2006). Adjuvant postoperative chemotherapy is often successfully performed in women affected with breast cancer, with the aim of increasing the survival rates (Clarke et al. 2005). Conversely, postoperative chemotherapy in canine patients is not routinely

used (Sleeckx et al. 2011). More often, chemotherapy is only suggested in cases of advanced disease with distant metastases or inoperable primary tumours (Karayannopoulou & Lafioniatis 2016).

Human breast cancers and canine mammary tumours share many features (Alvarez 2014; Liu et al. 2014), including histological and clinical similarities (Ranieri et al. 2013; Liu et al. 2014), comparable age distribution between species and malignancy rates (Queiroga et al. 2011). In women and dogs, hormonal exposure (both exogenous and endogenous), tumour size, lymph node status and changes in expression of steroid receptors or suppressor genes have been identified as important factors influencing tumour aggressiveness and tendency for metastasis (Yamagami et al. 1996; Chia et al. 2005; Morris et al. 2009; Telli & Sledge 2015). For all of these reasons, in the last few years, canine tumours have been suggested as translational models of naturally occurring cancers in humans (Alvarez 2014). Based on the fact that dogs have a shorter lifespan and consequently canine tumours require a shorter time to metastasize, clinical canine patients affected with mammary tumours could be adopted as a natural model for the study of mammary tumour progression on a shorter time scale.

The possible influence of perioperative factors on metastatic spread is of growing interest, and the role of anaesthetic and analgesic techniques has been investigated by recent retrospective studies in human medicine (Forget et al. 2010; Snyder & Greenberg 2010; Forget & De Kock 2014). Some drugs, such as ketamine and thiopental, have been suggested to facilitate cancer spread. For instance, they increased the amount of viable tumour cells found post-mortem in rats' lungs after animals were injected with tumour cells and

subjected to anaesthesia with these agents (Melamed et al. 2003). Conversely, local anaesthetics and non-steroidal anti-inflammatory drugs might decrease cancer spread. They seem to inhibit the invasive ability of tumour cells (Mammoto et al. 2002) or to impede prostaglandin secretion by neoplastic cells, a cancer cell's mechanism for evading host cell-mediated immunity (Wojtowicz-Praga 2003). The choice of anaesthetic drugs and techniques may therefore have a central role in the management of oncological patients. Bitches affected with mammary cancer, indeed, may undergo anaesthesia not only for surgical procedures, but also for diagnostic and medical work-ups, being repeatedly exposed to anaesthetic drugs. Thus, an understanding of the effects of anaesthetics on canine tumour progression becomes fundamental for improving outcome of oncological veterinary patients.

An essential abnormality resulting in the development of cancer is the continual unregulated proliferation of neoplastic cells (Feitelson et al. 2015; Keibler et al. 2016). In fact, the study of tumour cell proliferation has been indicated as a fundamental component when assessing cancer initiation and propagation (Cooper 2000; Adan et al. 2016; Präbst et al. 2017). Previous research data are available concerning the effects of anaesthetic agents such as propofol and sevoflurane on cancer recurrence. The agent propofol is one of the most commonly used intravenous anaesthetics in veterinary and human medicine (Berry 2015). Propofol has been shown to exert anti-cancer properties, decreasing the proliferation rate of osteosarcoma cells (Ye et al. 2014) and inhibiting the proliferation rate of breast cancer cells *in vitro* (Siddiqui et al. 2005; Deegan et al. 2009). Propofol has been demonstrated to

increase apoptotic rate in non-small lung carcinoma, colon carcinoma and breast cancer human cell lines (Song et al. 2014). Furthermore, propofol has been proven to initiate the activation and promote the differentiation of T-helper cells, fundamental in the host's defence against tumour progression (Wada et al. 2007). Although several reports on propofol's anti-metastatic properties are available, the exact mechanisms through which propofol exhibits these activities remain incompletely understood. Conversely, *in vitro* (Ecimovic et al. 2013) and retrospective clinical trials (Wigmore et al. 2016) have shown that volatile agents like sevoflurane might have a pro-tumorigenic effect, facilitating the development of metastases of many solid tumours (Wigmore et al. 2016). However, it is unknown whether propofol or sevoflurane could facilitate tumour cell proliferation in canine cell lines.

In human medicine, interest has risen in exploring the effects of drugs on the expression of genes associated with tumour cell migration such as neuroepithelial transforming gene 1 (NET1). This represents a Ras homologue gene family, member A (RhoA)-specific guanine nucleotide exchange factor (GEF) and has a fundamental role in the ability of human breast adenocarcinoma cells to migrate and invade (Glicrease et al. 2009). To the best of the author's knowledge, the effects of propofol and sevoflurane on the expression of NET1 have not previously been investigated in canine cell lines.

The aim of this project was to evaluate the effects of different concentrations of clinically available propofol and sevoflurane formulations on cell proliferation and on the expression of NET1 in primary (CIPp) and metastatic (CIPm) canine mammary tumour cell lines *in vitro*.

2. REVIEW OF THE LITERATURE

2.1 Canine mammary tumours

Canine mammary tumours are one of the most common types of cancer, representing 50 - 70% of all tumours occurring in bitches (Vail & MacEwen 2000; Camacho et al. 2014). Data regarding the incidence of canine mammary tumours vary due to factors such as study design, geographical location, routine in performing ovariohysterectomy and composition of the analysed population in terms of age, breed, and body size (Grüntzig et al. 2016). A study from the United States found an annual incidence rate of 258 malignant mammary tumours per 100 000 intact female dogs (Dorn et al. 1968), while a Swedish study based on 80 000 mostly intact female dogs reported 111 both benign and malignant mammary tumours per 10 000 dog-years at risk (Egenvall et al. 2005). A study based on the Swiss canine cancer registry, which includes data from 1955 to 2008 identified adenomas/adenocarcinomas as the most common tumour, 55% of which were located in the mammary gland (Grüntzig et al. 2015). Data from Genoa, Italy, collected between 1985 and 2002 showed an incidence rate of 192 mammary tumours per 100, 000 dog-years, which was the highest for all tumour types (Merlo et al. 2008).

Several studies have shown that benign and malignant mammary tumours are not completely separate entities, instead being part of a biological and histopathological progressive carcinogenesis process with direct similarities to human breast cancer (Gilbertson et al. 1983; Benjamin et al. 1999; Sorenmo et al. 2009). Canine mammary tumours are a very heterogeneous group of

neoplasms showing different hormonal expression, grade of aggressiveness and histopathological features. According to the literature, from 40% to 70% of canine mammary tumours are malignant, and the simple carcinoma represents the most common histological type, followed by mixed and complex tumours (Salas et al. 2015; Vascellari et al. 2016).

2.1.1 Pathogenesis and risk factors

Although tumorigenesis is not fully understood, a correlation between dysregulation of growth factors or hormones and the development of canine mammary tumours has been indicated. In 2008, Queiroga and colleagues showed that progesterone increases endogenous growth hormone production stimulating local and systemic insulin-like growth factor 1 secretion. These findings suggest that growth hormones act as local growth factors favouring the development and maintenance of canine mammary tumours in an autocrine manner (Queiroga et al. 2008). In parallel, Klopfleisch and co-workers (2011) suggested that aberrant expression levels of several functional classes of genes (*e.g.* mediators of apoptosis, deoxyribonucleic acid (DNA) repair systems, proto-oncogenes, tumour suppressor genes and cell adhesion molecules) hint at the molecular mechanisms underlying the development of canine mammary tumours. These genes and the proteins they encode may represent new potential therapeutic targets. However, the authors declared that although canine mammary tumours are among the most common tumours of the dog, their molecular carcinogenesis is far from being understood (Klopfleisch et al. 2011).

It has been widely accepted that ovariohysterectomy at a young age decreases the incidence of mammary cancer dramatically. Schneider and colleagues (1969) found that female dogs neutered before the first oestrus had an incidence of mammary tumours of 0.5%. The incidence increased to 8% and 26% if dogs were neutered after the first or the second oestrus, respectively. The study showed no differences between female dogs ovariohysterectomised after the second oestrus and intact ones (Schneider et al. 1969). These findings suggest that the critical carcinogenic effects of ovarian hormones on the mammary glands are time-limited and occur mostly during pubertal growth and maturation (Sorenmo et al. 2003). In accordance, physiological hormonal fluctuations during pregnancy or pseudo-pregnancy, usually occurring after a few oestrus cycles, have not been found to increase the risk of canine mammary tumour development (Schneider et al. 1969; Taylor et al. 1976).

A number of studies have investigated the effect of administered hormones, such as progestin and oestrogen, under laboratory conditions, with the majority of the investigators concluding that steroid hormone treatment increases the risk of canine mammary tumour development (Giles et al. 1978; Concannon et al. 1981; Selman et al. 1995).

The risk for developing a mammary tumour greatly increases after 5 years of age and reaches its highest point at 9 to 13 years (Schneider et al. 1969; Yamagami et al. 1996; Merlo et al. 2008), with older individuals being more likely to develop malignant lesions (Sorenmo et al. 2009). The life expectancy of different breeds also influences the peak incidence age. Larger breeds, having a shorter mean lifespan, have been diagnosed at a younger age than

smaller breeds (Egenvall et al. 2005). In general, purebred dogs are more likely to develop canine mammary tumours. Within this group, smaller breeds have a threefold higher risk than larger ones (Grüntzig et al. 2016).

2.1.2 Histological classification

The histopathological heterogeneity of canine mammary tumours results in a variety of proposed classification systems. Table 1 shows an improved system published in 2011 (Goldschmidt et al. 2011) based on two earlier classifications from the World Health Organization in 1974 and 1999 (Hampe & Misdorp 1974; Misdorp et al. 1999). Simple carcinomas are neoplasms originating from a single type of cell (epithelial or myoepithelial), while complex carcinomas originate from two different types of cells (epithelial and myoepithelial together). Mesenchymal neoplasms arise from mesenchymal tissue.

Table 1: Histological classification by Goldschmidt et al. (2011).

Histological Classification of Canine Mammary Tumours	
MALIGNANT EPITHELIAL NEOPLASMS	• **carcinoma–*in situ*** • **carcinoma–simple** ○ tubular ○ tubulopapillary ○ cystic-papillary ○ cribriform • **carcinoma–micropapillary invasive** • **carcinoma–solid** • **comedocarcinoma** • **carcinoma–anaplastic**

	• **carcinoma arising from a complex adenoma/mixed tumour** →the benign counterpart is still detectable in the section • **carcinoma–complex type** →the epithelial component is malignant, and the myoepithelium is benign • **carcinoma and malignant myoepithelioma** →the epithelial and myoepithelial components are malignant • **carcinoma–mixed type** →the epithelial component is malignant; the myoepithelial mesenchymal component is benign; and the mesenchymal component is cartilage or bone • **ductal carcinoma** →malignant counterpart of ductal adenoma • **intraductal papillary carcinoma** →malignant counterpart of intraductal papillary adenoma
MALIGNANT EPITHELIAL NEOPLASMS — SPECIAL TYPES	• **squamous cell carcinoma** • **adenosquamous carcinoma** • **mucinous carcinoma** • **lipid-rich** (secretory) **carcinoma** • **spindle cell carcinoma** • **malignant myoepithelioma** • **squamous cell carcinoma–spindle cell variant** • **carcinoma–spindle cell variant** • **inflammatory carcinoma**
MALIGNANT MESENCHYMAL NEOPLASMS — SARCOMAS	• **osteosarcoma** • **chondrosarcoma** • **fibrosarcoma** • **hemangiosarcoma** • **other sarcomas**

CARCINOSARCOMA — MALIGNANT MIXED MAMMARY TUMOUR	
BENIGN NEOPLASMS	• **adenoma–simple** • **intraductal papillary adenoma** (duct papilloma) • **ductal adenoma** (basaloid adenoma) • **with squamous differentiation** (keratohyaline granules) • **fibroadenoma** • **myoepithelioma** • **complex adenoma** (adenomyoepithelioma) • **benign mixed tumour**
HYPERPLASIA / DYSPLASIA	• **duct ectasia** • **lobular hyperplasia** (adenosis) • **regular** • **with secretory activity** (lactational) • **with fibrosis**→interlobular fibrous connective tissue • **with atypia** • **epitheliosis** • **papillomatosis** • **fibroadenomatous change** • **gynecomastia**
NEOPLASMS OF THE NIPPLE	• **adenoma** • **carcinoma** • **carcinoma with epidermal infiltration** (Paget-like disease)
HYPERPLASIA/DYSPLASIA OF THE NIPPLE	• **melanosis** of the skin of the nipple

Criteria for malignancy are histological type, nuclear and cellular pleomorphism, mitotic index, occurrence of necrotic areas and invasion of the tissues surrounding the tumour or the lymphatic vessels. Malignant canine

mammary tumours are more frequently of epithelial origin, with tubular carcinomas being the most common ones within this group (Sorenmo 2003; Sorenmo et al. 2009). Benign lesions are usually adenomas, lobular hyperplasia and benign mixed tumours (Sorenmo et al. 2009).

Most of the available systems for predicting prognosis are based on a method used for human breast cancer by Elston and Ellis (1991). This system (Table 2; from Sorenmo et al. 2003) divides the tumours into grade I (low score, well-differentiated), grade II (intermediate score, moderately differentiated) and grade III (high total score, poorly differentiated), based on the morphological criteria shown in Table 3 (Sorenmo et al. 2003).

Table 2: Grading system based on the Elston and Ellis system, adopted for dogs (Sorenmo et al. 2003).

Grade of malignancy	
Total score	Grade
3 – 5 points	Grade I (low), well-differentiated
6 – 7 points	Grade II (intermediate), moderately differentiated
8 – 9 points	Grade III (high), poorly differentiated

Table 3: Morphological criteria for the Elston and Ellis grading system for human breast cancer, adopted for dogs (Sorenmo et al. 2003).

Criteria for grade of malignancy		
Tubule formation	Nuclear polymorphism	Mitotic rate (Mitoses/ 10 high power fields)
Tubule formation >75% of the specimen → 1 point	Uniform or regular small nucleus and occasional nucleoli → 1 point	0-9 → 1 point

Moderate formation of tubular arrangements (10-75% of the specimen) admixed with areas of solid tumour growth → 2 points	Moderate degree of variation in nuclear size and shape, hyperchromatic nucleus, and presence of nucleoli (some of which can be prominent) → 2 points	10-19 → 2 points
Minimal or no tubule formation (<10%) → 3 points	Marked variation in nuclear size and hyperchromatic nucleus, often with one or more prominent nucleoli → 3 points	>20 → 3 points

Various studies evaluating the prognostic value of this grading system found it to be reliable for canine mammary tumours, increasing the potential for canine mammary tumours to be used as a natural model for the study of human breast cancer (Misdorp 2002; Karayannopoulou et al. 2005; Clemente et al. 2010; Goldschmidt et al. 2011).

2.1.3 Clinical presentation

Mammary tumours are often found by the owner or are incidental findings made during routine physical examination performed by the veterinarian since dogs affected with mammary tumours are frequently presented as otherwise clinically healthy. The tumours are mostly located in the caudal fourth and fifth mammary glands, but they can occur anywhere along the mammary chain (Cassali et al. 2014). Over 60% of dogs with mammary tumours have more than one lesion, and therefore, thorough palpation of all mammary glands is recommended.

The inflammatory carcinoma is a clinical entity characterized by a very inflamed, painful, swollen mass, usually confused with a severe mastitis, with

a very high risk of metastasis and a survival time of less than 60 days (Vail & MacEwen 2000).

About 50% of malignant canine mammary tumours exhibit invasion of local blood or lymph vessels or have already metastasized at the time of surgery, with 16% showing systemic metastasis (Gilbertson et al. 1983; Sorenmo et al. 2003; Karayannopoulou & Lafioniatis 2016). Non-specific symptoms (*e.g.* lethargy, weight loss and weakness) may be observed in dogs with advanced metastatic diseases (Sorenmo 2003). Canine mammary tumours can spread via the lymphatic or blood vessel systems to regional and distant lymph nodes (Sorenmo et al. 2003). Metastasis to the regional lymph node is an early step in cancer recurrence and is usually followed by the development of distant metastases, mainly in the lungs, liver and bones (Kitchell & Loar 1997; Misdorp 2002). Distant metastases ultimately lead to the death of the dog (Misdorp & Hart 1979; Benjamin et al. 1999).

2.1.4 Staging and diagnosis

A widely used method to determine the clinical stage of canine mammary tumours is the modified T (tumour size), N (lymph node status) and M (metastasis) classification by Owen (1980) shown in Table 4.

Table 4: TNM (tumour size, lymph node status, metastasis) classification by Owen (1980).

Staging of canine mammary tumours			
Stage	Tumour size	Lymph node status	Metastasis
Stage 1	T1<3 cm	N0	M0
Stage 2	T2 3-5 cm	N0	M0

Stage 3	T3 >5 cm	N0	M0
Stage 4	Any	N1 (positive)	M0
Stage 5	Any	N0 or N1	M1 (distant metastasis)

Cytology is often used as a diagnostic tool in veterinary oncology, and cytological findings correlate well with histopathological diagnosis for numerous tumour types such as squamous cell carcinomas, soft tissue sarcomas, melanomas, mast cell tumours and osteosarcomas, among others (Griffiths et al. 1984; Allen et al. 1986; Stockhaus & Teske 2001; Stockhaus et al. 2003; Reinhardt et al. 2005). In women, preoperative cytological evaluation of breast cancer is used to determine the presence of malignancy and is considered a rapid and minimally invasive alternative to surgical biopsies (Cheung et al. 1987; Costa et al. 1993; Ciatto et al. 1997; Orell & Miliauskas 2005). In dogs, little data are available on the value of cytological examination of mammary tumours. The few studies report less favourable accuracy of cytological diagnosis for mammary tumours than for other tumour types, with lower sensitivity and specificity (Griffiths et al. 1984; Allen et al. 1986; Hellmén & Lindgren 1989; Stockhaus & Teske 2001). Reasons for this have been assumed to lie, in part, with the heterogeneous composition of mammary tumours (Misdorp 1979; Rutteman 2000). However, more recent reports describe improved accuracy of cytological examination for the diagnosis of mammary tumours (Simeonov & Simeonova 2006a; Simeonov & Simeonova 2006b; Simeonov & Simeonova 2007), with correlations between cytological and histological diagnosis varying between 81% and 93% for malignant and benign tumours, respectively (Simon et al. 2009). Nevertheless, since surgery remains the treatment of choice, histological evaluation after surgical excision

is common in practice and still more accurate than cytology (Allen et al. 1986; Cassali et al. 2007).

Staging should include complete blood count, blood biochemistry, urinalysis and three-view radiography of the thoracic cavity (Biller et al. 2016; Gundim et al. 2016). Additionally, abdominal ultrasound should be conducted, especially if distant metastatic disease is suspected. Computed tomography offers a more sensitive alternative for detecting pulmonary metastasis than radiography. However, while computed tomography is routinely used in human medicine, radiography remains the technique of choice in veterinary medicine due to its lower costs and higher availability (Nemanic et al. 2006; Otoni et al. 2010; Eberle et al. 2011).

2.1.5 Therapy

The surgical approach is the treatment of choice. Most studies comparing the prognostic effects of different surgical approaches could not find any differences between simple lumpectomy, regional mastectomy, mastectomy, chain mastectomy and staged bilateral mastectomies as long as all lesions were sufficiently removed (Schneider et al. 1969; Misdorp & Hart 1976; MacEwen et al. 1985; Yamagami et al. 1996; Chang et al. 2005). However, multiple nodules should be treated individually because of their possibly different histopathological classification (Benjamin et al. 1999).

Currently, decisions regarding the surgical approach need to consider all of the previously described factors such as location, clinical stage and the presence of co-morbidities. Nevertheless, the choice of the surgical approach is subjective. Commonly, it is recommended that the surgical excision should

entirely remove the tumour mass. In the case of different mammary masses, regional or chain mastectomy is then suggested. The surgical excision is questionable in the case of inflammatory carcinoma due to the diffuse microscopic extension of the disease and the high grade of early metastasis (Vail & MacEwen 2000; Yamauchi et al. 2012).

The benefit of concurrently performing ovariohysterectomy is controversial. Most studies have found no difference in survival rates of ovariohysterectomized dogs relative to intact dogs (Schneider et al. 1969; MacEwen et al. 1985; Yamagami et al. 1996; Philibert et al. 2003). However, some studies report survival benefits if ovariohysterectomy is conducted within the 2 years preceding tumour surgery and for certain types of canine mammary tumours (*e.g.* complex carcinomas; from Sorenmo et al. 2000; Chang et al. 2005).

Few studies have investigated the efficacy of systemic chemotherapy, which remains to be confirmed. The chemotherapeutic drugs traditionally used in dogs are 5-fluorouracil and cyclophosphamide (Karayannopoulou et al. 2001), doxorubicin and docetaxel (Simon et al. 2006) and gemcitabine (Marconato et al. 2008), but postoperative benefits in survival time were not clear in these studies.

Hormonal therapy with, for instance, oestrogen receptor antagonists (*e.g.* Tamoxifen), frequently used in humans, has not found an application in the veterinary field due to lack of information about molecular expression and high medical costs (Karayannopoulou & Lafioniatis 2016).

2.1.6 Prognosis

Tumour size, lymph node involvement and histopathological stage are the three most important factors associated with prognosis. Despite being part of the TNM staging system, tumour size has been recognized as an independent prognostic factor (Misdorp & Hart 1976; Bostock 1986; Yamagami et al. 1996) with lesions smaller than 3 cm in diameter having a significantly better prognosis compared with larger ones (Gilbertson et al. 1983). Similarly, lymph node status has prognostic value on its own (Kurzman & Gilbertson 1986). Comparably to human breast cancer, metastasis to the regional lymph node is considered to be one of the most relevant prognostic factors in the diagnosis of canine mammary tumours (Misdorp & Hart 1976; Fitzgibbons et al. 2000). Chang and colleagues (2005) suggested that tumour stage, tumour size and ovariohysterectomy status were significant prognostic factors associated with 2-year survival after surgery in dogs with malignant mammary tumours. Further, dogs with tumours ≥ 5 cm in diameter and dogs with tumours present for > 6 months prior to surgery had a higher risk of lymph node metastases (Chang et al. 2005).

Histopathological type also influences survival. In general, sarcomas have a poorer prognosis than carcinomas, with the exception of inflammatory carcinomas. Within carcinomas, malignancy increases from carcinoma *in situ*, to complex carcinoma, to simple carcinoma and to anaplastic carcinoma (Misdorp et al. 1999).

One of the current challenges is to identify molecular tools and relevant models that can predict the response and potential resistance to therapies (Nguyen et al. 2018). Queiroga and colleagues (2010) investigated the

relevance of the expression of cyclooxygenase (COX) 1 and 2 enzymes as prognostic values in canine mammary tumours. They evaluated the correlation of COX1 and COX2 expression with clinico-pathological elements (*i.e.* tumour size, histological type, necrosis, lymph node metastasis), disease-free survival time and overall survival (Queiroga et al. 2010). Subsequently, Nguyen and co-workers (2018) described invasive canine mammary tumours using human pathological criteria including immunohistochemical markers. The same authors tried to validate human pathological criteria as prognostic factors for canine patients' outcome. Clinico-pathological criteria, similar to those of human oncology, could therefore predict canine invasive mammary carcinomas' short disease course (Nguyen et al. 2018). In a subsequent publication, the same group of researchers reported that the current molecular classifications of human breast cancer, used for therapeutic decisions, could be applied to dogs. Furthermore, the authors stated that dogs are potent valuable spontaneous cancer models to test new therapeutic strategies for human breast cancer treatment (Abadie et al. 2018).

2.1.7 Canine mammary tumours as a natural model of human breast cancer

There is growing interest in canine mammary tumours as a natural model for human breast cancer. In particular, the canine simple carcinoma has been recognized as a potential natural model for human breast cancer due to its histological and clinical similarities to the human form of the disease (Ranieri et al. 2013; Liu et al. 2014).

The high incidence rates of mammary tumours in dogs and dogs' shorter lifespan speak in favour of conducting clinical trials in veterinary medicine rather than in human medicine. Age-specific incidence rates rise dramatically in women older than 40 years and in dogs older than 5 years, with a peak incidence age of 55 - 69 years in women and 9 - 13 years in dogs (Yamagami et al. 1996; Merlo et al. 2008; Dewis & Gribbin 2009). Taking the different lifespan of both species into account, the age distribution of the disease is comparable (Queiroga et al. 2011).

In both humans and dogs, it has been demonstrated that hormonal exposure plays an important role in tumour behaviour, especially in the epithelial component. In humans, oestrogen expression, found in 20 - 25% of cases, is associated with aggressive tumour behaviour and poor prognosis (Telli & Sledge 2015). In veterinary studies, important discrepancies were found in these data; the majority of larger and undifferentiated canine mammary tumours are less likely to express hormonal receptors, although the immunohistochemical methods vary between studies with a lack of standardization (Kabir et al. 2017; Silveira et al. 2017).

Body size as a risk and prognostic factor for human breast cancer is well-established (Greenberg et al. 1985). In particular, weight loss in early adult life, and not weight *per se*, has been demonstrated to decrease the risk of breast cancer diagnosed at an early age (Kotsopoulos et al. 2005). In dogs, although not without controversy, juvenile obesity seems to increase the risk of canine mammary tumour (Sonnenschein et al. 1991; Pérez-Alenza et al. 1998). A thin body conformation at 9 - 12 months of age, indeed, was shown

to reduce the risk of mammary tumours among neutered dogs by 99%, and among entire dogs by 40% (Sonnenschein et al. 1991).

In both species, tumours of epithelial origin are most commonly found (Sorenmo 2003; Weigelt et al. 2008; Akiyama & Horii 2009; Sorenmo et al. 2009). Carcinomas represent 69% of malignant lesions in dogs (Salas et al. 2015) and up to 95% of malignant lesions in humans (Vinay et al. 2010). Histological grading correlates with prognosis (Karayannopoulou et al. 2005; Rakha et al. 2008; Goldschmidt et al. 2011). Tumour size, lymph node status and overall TNM stage are important prognostic factors in canine mammary tumours and human breast cancers (Misdorp & Hart 1976; Gilbertson et al. 1983; Shek & Godolphin 1988; Hellmén et al. 1993; Yamagami et al. 1996).

2.2 Effects of perioperative period on cancer recurrence

The role played by anaesthetists in avoiding postoperative surgical site infections and in facilitating postoperative healing has been acknowledged (Nortcliffe & Buggy 2003; Bentov & Reed 2014). However, the role of the anaesthetist in ameliorating long-term outcome after cancer surgery remains to be elucidated (Sessler et al. 2008; Forget et al. 2010). Exadaktylos and colleagues (2006) proposed an analogy between prolonged healing of the surgical site and the development of postoperative metastasis, identifying the perioperative period as a critical time for deleterious long-term consequences for the patient. The general understanding of the relationship between tumour biology and host defences is the basis of the idea that interventions occurring

during the perioperative period will affect long-term cancer patient outcome (Schmidt et al. 2008; Milsom et al. 2013).

2.2.1 Cancer metastatic mechanisms

The likelihood of development of tumour metastasis depends on the equilibrium between the metastatic potential of the tumour and the anti-metastatic defences of the host (Shakhar & Ben-Eliyahu 2003). The so-called "seed and soil" hypothesis represents one of the theories that explains how a tumour acquires metastatic potential (Fidler 2003). It depicts a gradual increment of the primary tumour's dimensions, during which time the supply of nutrients is initially met by mechanisms of diffusion, later requiring neovascularization. Angiogenic factors are produced and secreted, and a system of capillaries rises from adjacent tissues. Cancer cells subsequently invade the host circulation, most commonly through the lymphatic vessels. The host immune defences, described below, will destroy most of these cells. However, surviving tumour cells will become lodged in the capillaries of distant organs, then extravasate, multiply, and eventually build their own blood supply, becoming a micrometastasis (Fidler 2003; Langley & Fidler 2011).

A process called epithelial-to-mesenchymal transition (EMT), first documented by Elizabeth Hay (1995), facilitates alterations in cell phenotype, which are critical in early embryonic morphogenesis. Epithelial cells are immobile, firmly connected to neighbouring cells by junction structures (*e.g.* adherent, tight and gap junctions) and have an apical-basal polarity made possible by their connection to the basement membrane. Mesenchymal cells,

on the other hand, showing a front end-back end polarity, form only temporary contacts with neighbouring cells and have the ability to invade and move through the extracellular matrix as individual cells (Hay 1995; Kalluri & Weinberg 2009; Heerboth et al. 2015; Brabetzl et al. 2018; Roche 2018).

The EMT not only occurs during embryonic development, but is also involved in tissue repair in the adult organism (Acloque et al. 2009), and, more recently, its role has been acknowledged in tumour progression (Trimboli et al. 2008; Heerboth et al. 2015). Therefore, a classification into three subtypes of EMT has been established: EMT type 1 is involved in implantation, embryonic formation and organ development; EMT type 2 is involved in repair processes following tissue destruction and chronic inflammation; EMT type 3 enables epithelial tumour cells to invade and migrate (Kalluri & Weinberg 2009; Zeisberg & Neilson 2009).

2.2.2 Role of the immune system in cancer recurrence

Neoplastic cells are the result of an evolving process during which they erratically mutate and experience selection. Originally, tumour cells are blandly antigenic, provoking almost no response from the immune system. This stage has been recognised as a phase of uncontrolled proliferation and mutation (Shakhar & Ben-Eliyahu 2003). As mutation progresses further, cancer cells become more antigenic. This represents a stage of identification, elimination and selection of immune-resistant cells. Finally, the tumour cells mature mechanisms to escape from the immune response of the host. The outcome and duration of these stages may depend on factors which include

the spatial cancer location, molecular mechanisms involved in transformation from normal to transformed cells, and the inherent genetic factors of the immune system (Mittal et al. 2014).

The cardinal host defence against metastatic progression is an intact cell-mediated immune system (Shakhar & Ben-Eliyahu 2003). Natural killer (NK) cells are a subpopulation of lymphocytes that spontaneously recognise and destroy neoplastic cells, representing the primary defence against cancer cells (Biki et al. 2009). Interleukin (IL)-2 and interferon-γ (IFN-γ) are important activators of NK cells (Andersen et al. 1998). Several studies demonstrate a counter association between NK cell activity at the time of surgical tumour excision and the advancement of metastatic disease (Brittenden et al. 1996).

It is recognised that the cytotoxic activity of T-cells is another key factor in defence against cancer. Indeed, human patients presenting with elevated cytotoxicity against their primary lung cancer have been seen to undergo complete remission, while none of the patients presenting in the same study with low cytotoxicity survived (Uchida et al. 1990).

The importance of an intact cell-mediated immune system can be shown in the context of organ transplant receivers, in which immunosuppressive therapy is commonly needed and appears to promote the development of metastasis (Penn 1993). Importantly, the primary tumour is not destroyed by cell-mediated immunity. Nevertheless, cell-mediated immunity may eradicate the “minimal residual disease”, a term used to describe the cancer cells that remain in the body after curative resection of the primary mass.

2.2.3 Suppression of the immune system in the perioperative period

As already stated, the ultimate development of neoplastic cells that can elude host cellular immunity is a critical step in the formation of metastasis. Major surgery has been demonstrated to suppress cell-mediated immunity for several days after surgery (Shakhar & Ben-Eliyahu 2003). In the perioperative period, an increase can be observed in the release of cytokines such as IL-10 that negatively affect cell-mediated immunity. Conversely, a reduction in the release of cytokines that favour cell-mediated immunity such as IL-2, IL-12 and IFN-γ usually occurs (Snyder & Greenberg 2010). The result is a diminished quantity of circulating NK cells, T helper cells, dendritic cells and cytotoxic T-lymphocytes (Buggy & Smith 1999). A peak in immunosuppression is said to occur on day 3 after surgery (Coffey et al. 2003), and this may be a window of opportunity for the minimal residual disease to grow and spread.

In humans, the psychological stress associated with surgery can contribute to perioperative immunosuppression (Andersen et al. 1998). In parallel, it has been shown that social conflict and aberrant fear conditioning (*e.g.* swim stress) impair the activity of NK cells in rats, augmenting lung cancer metastatic potential (Stefanski & Ben-Eliyahu 1996).

However, stress is not only psychological, but also physical. Acute pain can suppress NK cell activity (Shavit et al. 1987; Sacerdote et al. 1994). A rat model demonstrated that optimizing postoperative pain management might attenuate the postsurgical inhibition of the host's anti-tumour defence mechanisms, including NK cells (Page et al. 2001).

A decrease in body temperature to 30°C in rats was shown to impair the activity of NK cells and to reduce metastasis resistance (Ben-Eliyahu et al. 1999). However, hypothermia of 33 - 35°C was not demonstrated to cause similar consequences (Melamed et al. 2003). In humans, the immunosuppressive effects of abdominal surgery worsened when associated with mild hypothermia, most likely through the stimulation of glucocorticoid release and sympathetic nervous system response (Belin et al. 1998).

Perioperative allogeneic blood transfusion is another important factor that might be associated with an increased risk of tumour recurrence due to the immunosuppressive effect of allogeneic blood (Blajchman 1999).

2.2.4 Effects of drugs

2.2.4.1 Intravenous anaesthetic agents

The possible consequences of the use of anaesthetic drugs on the activity of host immune system have been evaluated using *in vitro* and animal models in addition to human studies (Snyder & Greenberg 2010).

In a rat model, thiopental and ketamine were shown to augment the quantity of viable lung cancer cells, while propofol and diazepam did not show a similar effect (Melamed et al. 2003). In the same study, the activity of NK cells was significantly reduced by thiopental and ketamine, but, again, not by propofol. Nevertheless, all three anaesthetic agents induced a substantial reduction in the quantity of NK cells compared with controls.

There are studies, which advocate propofol as an inhibitor of neoplastic cell proliferation, supporting the theory that propofol may decrease the incidence of cancer recurrence and prolong survival time of human patients with cancer

(Deegan et al. 2009; Ye et al. 2014). Indeed, propofol and propofol conjugates inhibited cells growth *in vitro* when applied to breast cancer cell cultures at clinically relevant concentrations (Siddiqui et al. 2005; Ecimovic et al. 2014). However, findings regarding the effects of propofol on tumour cell progression are controversial. For instance, propofol was able to induce proliferation in gallbladder cancer cells (Zhang et al. 2012) and neuroblastoma cells (Wu et al. 2011) *in vitro*. The effects of propofol on breast cancer cells have been extensively studied (Siddiqui et al. 2005; Ecimovic et al. 2014; Lim et al. 2018). To the authors' knowledge, the effects of propofol on canine mammary tumour cells have never been investigated. Currently, the lack of such a study does not allow comparisons between species on this topic. More evidence regarding the effects of propofol on canine mammary cancer cells would improve the understanding of the possible role of canine patients as a natural model for the study of human breast cancer. Nevertheless, the evaluation of propofol effects on canine mammary tumour cell proliferation, could give an initial indication for its advantageous clinical use in canine patients affected with cancer. Following these considerations, the authors decided to investigate with the present study the effects of propofol on canine mammary tumour cells proliferation and NET1 gene expression *in vitro*.

2.2.4.2 Inhalant anaesthetic agents

Multiple *in vitro* studies utilizing inhalant anaesthetic agents have shown effects that may have some relevance to cancer patients (Brand et al. 1997; Schlagenhauff et al. 2000; Benzonana et al. 2013; Zhang & Shao 2016; Lim et al. 2018). For instance, halothane and isoflurane impaired the stimulation

of NK cell cytotoxicity by IFN-γ in mice (Markovic et al. 1993). Along the same line, the exposure to nitrous oxide was linked with hastening of lung and liver metastasis growth, in a mouse cancer model (Shapiro et al. 1981). Finally, sevoflurane was shown to increase proliferation, migration and invasion of human breast cancer cells (Ecimovic et al. 2013). However, a more recent *in vitro* analysis documented the effects of blood sampled from human patients undergoing sevoflurane- or propofol-based anaesthesia on breast cancer cells apoptosis, NK cell activity and cytotoxic T-lymphocyte function. No differences were observed between treatments (Lim et al. 2018).

Data from clinical trials often appear more difficult to interpret due to the presence of confounding variables. For instance, during surgical interventions patients are usually exposed to several different medications such as sedatives, anaesthetics, analgesics and antibiotics among others (Exadaktylos et al. 2006; Wigmore et al. 2016).

Recently, sevoflurane has been utilized as a representative of the inhalant anaesthetic category in studies evaluating the effects of these drugs on cancer recurrence (Ecimovic et al. 2013; Wigmore et al. 2016; Lim et al. 2018; Hong et al. 2019). For this reason and to facilitate comparisons between studies, sevoflurane was chosen to represent inhalant anaesthetics in the present study.

2.2.4.3 Local anaesthetic drugs and locoregional anaesthesia

Lidocaine administered at clinical doses was shown to inhibit proliferation and invasive ability of human cancer cells *in vitro* (Mammoto et al. 2002; Sakaguchi et al. 2006). Ropivacaine suppressed, *in vitro*, the growth of cancer cells

derived from human patients affected with proliferative colon adenocarcinoma in a dose-dependent manner (Martinsson 1999). The concentrations applied were within the range of the therapeutic concentrations obtained inside the colon of patients treated rectally with ropivacaine. Conversely, in the same study, lidocaine, hydrocortisone and 5-aminosalicylic acid were found to be less potent than ropivacaine in inhibiting proliferation of human colon adenocarcinoma cells (Martinsson 1999).

In a retrospective study, patients undergoing open prostatectomy and receiving a local anaesthetic epidurally combined with general anaesthesia had a 57% lower risk of cancer recurrence than patients receiving general anaesthesia plus opioids (Biki et al. 2008). Another retrospective study, which included 129 patients undergoing mastectomy and axillary clearance, showed a substantial reduction in tumour recurrence and metastasis when breast cancer surgery was performed with paravertebral anaesthesia and analgesia compared with general anaesthesia and morphine analgesia (Exadaktylos et al. 2006).

Some multicentre randomized controlled trials have been initiated in human medicine. However, there is still a lack of published prospective studies explicitly intended to look at the effect of regional anaesthesia on the outcome of human or veterinary cancer patients. The presumptive ability of regional anaesthesia to improve long-term outcomes following cancer surgery is commonly linked to three diverse mechanisms (Sessler 2008). First, the immunosuppressive effect of surgery could be attenuated by regional anaesthesia (O'Riain et al. 2005). In fact, in humans undergoing breast surgery, paravertebral nerve blocks performed with bupivacaine 0.25% were

shown to prevent neuroendocrine stress response (O'Riain et al. 2005). Second, patients who receive regional analgesia should have lower opioid requirements, minimizing the negative impact of opioids on the immune system in terms of inhibition of cell-mediated immunity and host anti-tumour defences (Moller et al. 2007). Third, regional anaesthesia should reduce the amount of both inhalant and injectable general anaesthetics required during surgery itself (Snyder & Greenberg 2010).

2.2.4.4 Opioids

Perioperative and chronic administration of opioids has been proven to suppress humoral and cell-mediated immunity in *in vitro* and in human clinical studies (Sacerdote 2008; Sessler 2008; Liang et al. 2016; Zajączkowska et al. 2018).

Morphine suppresses rat and human NK cell cytotoxicity in a dose-dependent manner (Beilin et al. 1989; Saurer et al. 2006; Boland & Pockley 2017) and increases angiogenesis, promoting breast tumour growth in mice (Gupta et al. 2002). Comparably, fentanyl suppresses postoperative NK cell cytotoxicity in humans (Beilin et al. 1996; Boland & Pockley 2017), and patients receiving higher doses showed longer suppression times. In a study from Yeager and colleagues (1995), 23 healthy volunteers underwent continuous exposure to morphine for 36 hours, including an intravenous infusion of morphine of 0.05 mg kg^{-1} loading dose followed by a constant rate infusion of 0.03 mg kg^{-1} h^{-1} for 24 hours. Before conclusion of the morphine infusion, 5 participants were withdrawn from the study due to morphine-related side effects, such as anorexia, nausea, vomiting and pruritus. Peripheral blood

was sampled from the rest of the participants at five measurement times before, during and after morphine exposure. The results showed a significant suppression of NK cell cytotoxicity, persisting for 24 hours after termination of morphine infusion (Yeager et al. 1995).

In contrast, a beneficial effect has been demonstrated when morphine was administered preoperatively in rats undergoing laparotomy (Page et al. 1998), suggesting a possible role for preoperative morphine administration in reducing surgery-induced stress and metastatic potential.

Interestingly, tramadol, which in addition to its action at opioid receptors has noradrenergic and serotonergic effects, stimulates NK cells activity, in both rodents and humans (Gaspani et al. 2002).

2.2.4.5 Non-steroidal anti-inflammatory drugs

Neoplastic cells release prostaglandins and thereby evade host cell-mediated immunity (Wojtowicz-Praga 2003; Allaj et al. 2013). Non-steroidal anti-inflammatory drugs inhibit prostaglandin synthesis via inhibition of the COX enzymes, demonstrating some degree of anti-tumorigenic properties (Leahy 2002; Menter & DuBois 2012). In women with breast cancer, an overexpression of COX-2 enzymes has been shown (Arun & Goss 2004; Lucci et al. 2005). Furthermore, women taking COX-2 inhibitors for a long time experienced a decreased incidence of breast cancer and improved metastatic biomarkers (Harris et al. 2006).

2.3 *In vitro* cancer research

"In vitro" comes from the Latin term "in glass". The term refers to studies of biological properties that are done in a test tube (*i.e.* in a glass vessel) rather than in a human being or in an animal (Ramesh et al. 2016). *In vitro* studies allow scientists to isolate specific cells, bacteria and viruses and evaluate them in isolation from the whole organism. Unfortunately, this means that sometimes results obtained during *in vitro* studies do not translate to *in vivo* scenarios (Mak et al. 2014).

Relative to *in vivo* studies, *in vitro* studies are substantially faster, less expensive and provide fewer ethical concerns (Polli 2008). *In vitro* research is essential for making medical advances since it allows a substance to be studied without exposing human beings or animals to the possible side effects of a new drug (Gentile et al. 2017). Importantly, *in vitro* studies enable more rapid development of new treatments. Many drugs can be investigated simultaneously in a large number of sampled cells and only those that appear to be efficacious go on to *in vivo* studies (Hulkower & Herber 2011; Stoddart et al. 2011).

2.3.1 Canine mammary tumour cell culture

Cancer research has benefited immensely from the use of cancer cell lines (Präbst et al. 2017), which simultaneously provide a uniform sample and a large number of single units to analyse (Ramesh et al. 2016). Furthermore, cancer cell lines are suitable for study in a variety of ways and for a variety of applications (Stoddart et al. 2011).

Canine mammary tumour cell lines can be established from canine primary or metastatic cancer tissues, the samples of which can be obtained after surgical removal or euthanasia of canine patients clinically and histopathologically diagnosed with mammary cancer (Holliday & Speirs 2011; Yamauchi et al. 2012). After sampling, tumour tissue is processed for histopathological confirmation of the previous diagnosis. Tumour tissue is usually fragmented, washed and disaggregated. Thereafter, the disaggregated tissue suspension is centrifuged and the pelleted cells are re-suspended in a suitable cell culture medium and maintained in a standard condition *(i.e.* 37°C in a humidified atmosphere with 5% carbon dioxide; from Uyama et al. 2006; Caceres et al. 2015; Osaki et al. 2016; Gentile et al. 2017). Accurate identification of cell lines is crucial during cell line development to avoid the risks of using misidentified cells (Cheung et al. 2014).

Nowadays, the canine mammary tumour cell lines used for culture in *in vitro* studies are numerous and readily available (Norval et al. 1984; Osaki et al. 2016; Gentile et al. 2017).

In the present study, we utilized two cell lines of canine mammary tubular adenocarcinoma established by Uyama and colleagues (2006). We choose this pair of cell lines because deriving from the same individual, but presenting different evolutionary states. In fact, one cell line originated from the primary mass, while the other was obtained from a metastatic regional lymph node (Uyama et al. 2006). The aim was to verify whether the same cells in different evolutionary states would respond in dissimilar ways when exposed to the same drugs.

2.3.2 Tests for cancer research

Cancer cell lines provide a convenient representation of cancers and can undergo several assays to investigate different aspects of cancer biology (Ramesh et al. 2016). Cells are routinely cultured in 6-, 12-, 24- and 96-well plates. Agents can be studied in several concentrations and in duplicate, triplicate or larger multiples to obtain meaningful and reliable results (Van Meerloo et al. 2013).

Assays in general are performed for two main reasons: to ascertain the effects of a physical or chemical agent on the cells or to understand the behaviour of cells under different conditions (Ramesh et al. 2016). Available approaches to individuate the effects of a physical or chemical agent include testing cells for cytotoxicity, genotoxicity, proliferation, migration, invasion and changes in signalling pathways that can affect cancer cells when exposed to a specific agent (Korch et al. 2012; Yang et al. 2014). The assays can be performed on cancer cells with the aim of studying individual cells or to analyse the entire cell population to shed light on the physiology or behaviour of cells under different conditions (Borowicz et al. 2014).

2.3.2.1 Proliferation

Some *in vitro* assays are performed to quantify proliferation, which reflects cellular responses to various stimuli. These techniques help in monitoring cell division, the number of cells generated over time, the proliferative ability of cells and DNA synthesis (Präbst et al. 2017). Traditionally, proliferative assays are performed in drug testing to assess the effects of drugs on cell proliferation. Conventionally, cell proliferation has been assessed by its DNA-

synthesizing capacity. With this technique, cell lines are incubated for a few hours with 3H-thymidine (a radioactive label). Proliferating cells integrate the radioactive label during the process of DNA synthesis, which can be visualized and quantified using a scintillation counter (Adan et al. 2016).

The MTT or 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide cell proliferation assay is widely used for measuring cell proliferation with respect to cellular metabolism (Präbst et al. 2017). In fact, the MTT assay specifically evaluates cellular mitochondrial activity as an indicator of cellular viability. This technique involves a reduction of tetrazolium salts by mitochondrial dehydrogenase enzymes, generating reduced nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate. The resulting metabolite formazan (purple colour) can be quantified by spectrophotometry, measuring the absorbance at *circa* 570 nm. This chemical reaction yielding a purple product is displayed only in metabolically active cells, thereby eliminating non-proliferating cells (Tada et al. 1986; Weichert et al. 1991; Präbst et al. 2017). The MTT colorimetric assay does not involve the use of radioactive markers and for this reason is easy to perform (Van Meerloo et al. 2011).

Moreover, cell proliferation can be achieved by measuring the amount of adenosine triphosphate (ATP) generated in the cells (Adan et al. 2016). This technique is based on the concept that dead and non-proliferating cells generate a negligible amount of ATP. The luciferase enzyme generates light in the presence of ATP, which can be efficiently detected by a luminometer. This assay is proficiently used to screen a large number of samples.

Finally, cellular proliferation can be analysed with the use of a chemical reaction involving the conversion of the non-fluorescent dye resazurin (blue) to the fluorescent dye resorufin (pink) in a reducing growth medium. The fluorescent signal is then measured at selected wavelengths (570 and 600 nm).

The rationale for selecting a method of analysis depends on several factors such as the experimental setting, the type of drugs used and the biological effects that the scientists wish to investigate. The MTT proliferation assay is the most commonly performed test for studying proliferation of cells exposed to drugs due to its simplicity of use (Rai et al. 2018). For this reason, the MTT method was chosen to investigate the effect of propofol and sevoflurane on canine mammary tumour cells proliferation in the present study. This choice allowed the authors to compare their data with previous studies on the topic of cancer cell proliferation in response to drug exposure.

2.3.2.2 Migration

For the *in vitro* analysis of cell migration, commonly used assays include the Boyden chamber assay, the scratch wound-healing assay and more recently the microfluidic assay (Chen & Nalbantoglu 2014).

The Boyden chamber assay (trans-well migration assay, filter membrane migration assay or chemotaxis assay) can be performed quickly and employs chemoattractants (*e.g.* serum). A microporous membrane is utilized, which divides the culture well into two parts. In one part, usually the uppermost, the cells are cultured, while the lower compartment contains chemotactic agents

(Chen 2005). The migratory cells can be quantified using a plate reader or by staining of the membrane.

In the wound-healing assay, cells are maintained as monolayer cultures. At 80% confluence, cells are exposed to an inhibitor of cell division. After an adequate incubation time, a scratch (wound) is made using a sterile micropipette tip. The wound is observed under phase contrast inverted microscopy at various time intervals and its width is measured. To visualize the migration of cancer cells, fluorescent microscopic techniques can be used, and documentation of the scratch can be made using time-lapse imaging. The major drawback of the scratch assay is the time taken to perform the assay (Cory 2011).

The microfluidic assay has the potential to be physiologically relevant for testing the migratory capabilities of cells when exposed to a drug. The device offers dual ports, where one port is used to introduce cells and the other port is used to deliver the drugs. The cells once introduced into the device attach at the port bottom, and the drug delivered through the port at the other end of the chamber sets up a gradient. The cells are imaged to visualize migration (Hulkower & Herber 2011).

2.3.2.3 Invasion

One extremely important property of malignant cells is their invasive growth pattern. This ability allows cancer cells to leave the compartment to which they are normally restricted, to gain access to connective tissues and vessels and to complete the initial phase of the process of metastasis (de Both et al. 1999; Hall & Brooks 2014). Invasive properties are in part reflected by cell shape. In

fact, epithelioid cells appear less invasive than spindle-shaped ones (Sommers et al. 1992; Boyer et al. 1996).

To test the invasive capacity of cancer cells, numerous assay systems have been developed. They include the matrigel assay, the invasion in layers of confluent cultured fibroblasts technique and the chicken heart tissue invasion assay (Hall & Brooks 2014).

The matrigel assay uses the confrontation of cancer cells with porous membranes coated with components of the basement membrane to assess their ability to penetrate the coated filter in a specific time frame (Albini et al. 1987).

The invasion in layers of confluent fibroblasts technique is performed using a co-culture of tumour cells with a confluent layer of fibroblasts to monitor the invasion of tumour cells into the layer (Fabra et al. 1992). Non-invasive cells remain on the surface of the confluent fibroblast layer, whereas invasive cells invade, pushing away and replacing the layer of fibroblasts.

To perform the chicken heart assay, chicken hearts are co-cultured with cancer cells for some days in a suspension culture. The invading cancer cells can be subsequently detected in histological heart sections stained with haematoxylin and eosin (Mareel et al. 1979).

2.3.2.4 Changes in gene expression

Gene expression studies on cell lines provide genetic information associated with cellular functions such as proliferation, apoptosis, cell division, adhesion and intercellular communication (Ramesh et al. 2016; Caracausi et al. 2017). Rather than DNA, messenger ribonucleic acid (mRNA) is widely used as the

study material to understand the alterations in gene expression. This is due to the possibility of post-transcriptional changes in the sequence of DNA, which will be predominantly reflected in the mRNA.

Protein estimation and detection can also predict gene expression changes at translational levels, and the Western blot is a common technique used for detection of proteins directly associated with genetic alterations (Harris 2015; Ramesh et al. 2016).

2.3.2.4.1 Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was developed in the 1990s as a modified version of the PCR method introduced by Kary Mullis and colleagues in 1986 (Mullis et al. 1986; Higuchi et al. 1992). While both methods amplify a specific DNA sequence more than a billion-fold, real-time PCR simultaneously detects the investigated DNA segment, eliminating the subsequent detection steps, such as agarose-gel electrophoresis, Southern blot or PCR-ELISA (enzyme-linked immunosorbent assay), necessary in conventional PCR. This makes real-time PCR less labour-intensive and less susceptible to cross-contamination (Mackay et al. 2002; Wilhelm & Pingoud 2003; Houghton & Cockerill 2006).

Before PCR was introduced, the ability to detect a specific DNA sequence was limited by its initial quantity in the sample investigated. Polymerase chain reaction eradicates this limitation by producing numerous copies of the targeted DNA segment using enzymes called polymerases. Polymerases are present in all living cells and their task is to copy, proofread and correct genetic material (Powledge 2004). *Taq*-polymerase (from *Thermus aquaticus*) is a

frequently used enzyme in PCR (Valasek & Repa 2005). Primers, two strands of nucleotides complementary to the sequence of nucleotides on either side of the sought DNA section, tell the polymerases where to begin the synthesis. They are individually created in the laboratory or acquired from commercial suppliers.

Generally, PCR involves three steps: 1) denaturation → separating the double-stranded DNA (dsDNA) by heating to over 90°C, 2) annealing → binding of the primers and 3) DNA synthesis by polymerases (Figure 1; from www.thermofisher.com). In real-time PCR, the DNA segment under investigation, is amplified and detected at the same time by measuring fluorescence changes.

There are different detection formats available, which differ in their method of generating a fluorescence signal proportional to the amplification products by using different so-called probes. The earliest and simplest way is to use dsDNA-specific dye (*e.g.* ethidium bromide, YOYO-1 and SYBR-green). In contrast to other detection assays, the fluorophores bind sequence-independently to all dsDNA, making the design of a specific probe obsolete (Powledge 2004).

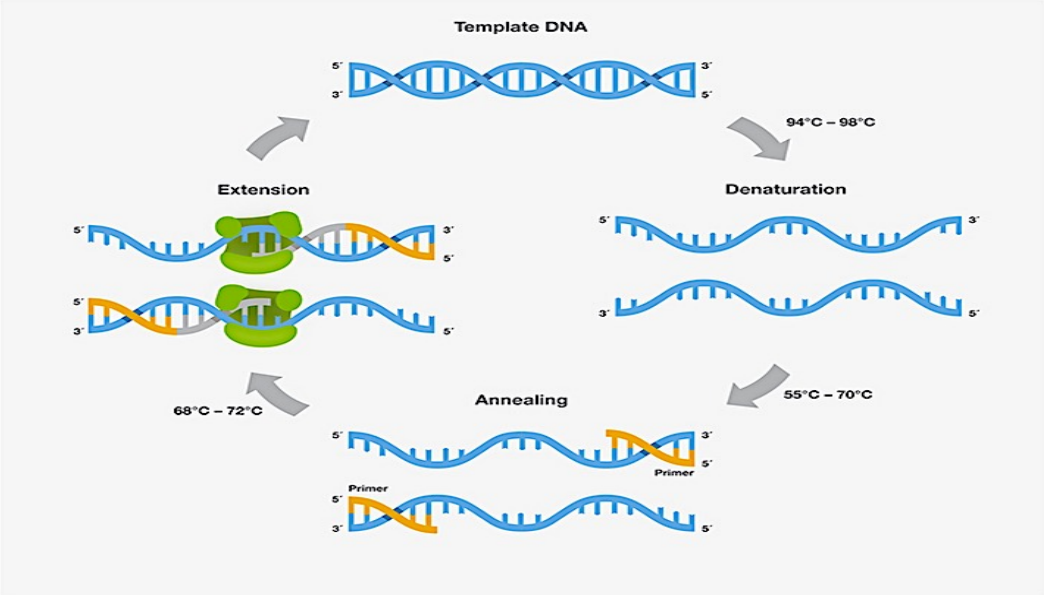


Figure 1: Individual steps of PCR: 1) denaturation, 2) annealing, 3) extension (www.thermofisher.com).

2.3.2.4.2 Quantitative assay for gene expression (quantitative PCR)

The quantitative polymerase chain reaction (qPCR) technique has been incorporated into laboratory routine by various researchers working on cancer cell lines. This technique is able to quantify the number of copies of a target gene in a specific sample or cell line treated with a specific molecule. Such studies are also useful for analysing various metabolizing genes that indicate drug effects (AbuHammad & Zihlif 2013).

The basis of this assay is a PCR performed on complementary DNA (cDNA), where the generated amplification curves are analysed to estimate the initial template amount of the specific gene (*i.e.* target gene). The amplification curve consists of three phases: 1) the initial lag phase, 2) the exponential phase and 3) the plateau phase (Figure 2; from Rodriguez-Lazaro & Hernandez 2013). In the exponential phase, the fluorescence signal

increase correlates directly with the product increase. The estimate of the initial template amount is based on the number of cycles needed for the signal to reach an arbitrary threshold, the so-called cycle threshold (Ct) value, shown as the intersection point or crossing point of the signal curve in its exponential phase with the threshold (Figure 2). The Ct value is indirectly proportional to the template amount of the target gene (Wilhelm & Pingoud 2003).

The NET1 gene is related with tumour cell migration. The study of NET1 gene expression through qPCR was performed in the present project to evaluate possible changes in cell behaviour associated to the exposure of canine mammary cancer cells to propofol and sevoflurane.

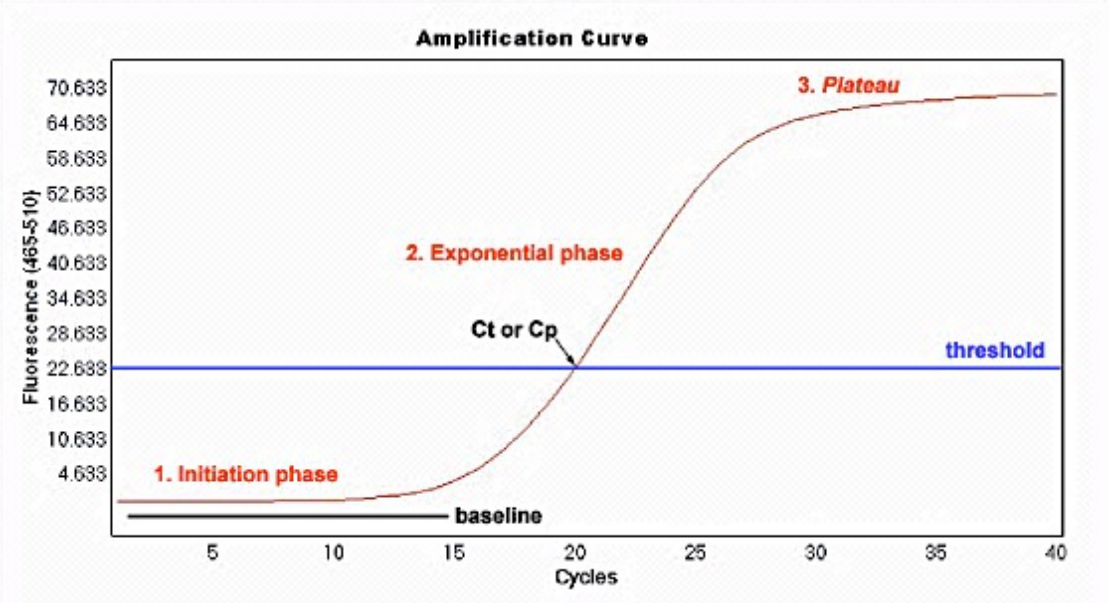


Figure 2: Polymerase chain reaction amplification curve comprising three phases: 1) the initial lag phase, 2) the exponential phase and 3) the plateau phase. Ct = cycle threshold; Cp = crossing point (Rodriguez-Lazaro & Hernandez 2013).

2.4 NET1 protein

The NET1 protein is a RhoA-specific GEF and was first identified as an oncogene in neuroepithelioma cells (Chan et al. 1996). The proteins encoded by NET1 gene primarily interact with RhoA, which is a member of the Rho family of small guanosine triphosphate hydrolase enzymes (GTPases; from Alberts et al. 1998). The Rho GTPases function as molecular switches of signalling cascades controlling a variety of cell functions, including actin cytoskeleton organization, cell polarity, cell adhesion and cell motility (Hall 1998; Bishop & Hall 2000; Bokoch 2000; Etienne-Manneville & Hall 2002; Stamnes 2002; Begum et al. 2004; Jaffe & Hall 2005).

The GTPases change between their active and inactive conformation by either binding guanosine triphosphate (GTP) or guanosine diphosphate (GDP). Several proteins regulate this fluctuation. Guanine nucleotide exchange factors catalyse the release of GDP and the binding of GTP, thus activating Rho GTPases. The GTPase-activating proteins increase the intrinsic GTP hydrolysis activity in order to enter the inactive state, and guanine nucleotide dissociation inhibitors maintain a cytosolic reserve of GDP-bound Rho proteins ready for activation (Rossman et al. 2005; Bos et al. 2007).

In cancer research the NET1 protein has a fundamental role in the organization of actin filaments in the cytoskeleton, and its overexpression has been demonstrated to increase the ability of human breast adenocarcinoma cells to migrate and invade (Glicrease et al. 2009).

2.4.1 RhoA in cancer biology

The RhoA protein family and the NET1 gene are especially noteworthy in the context of EMT. Figure 3 shows EMT during tumour dissemination and emphasizes the necessity of the reverse mechanism, the so-called mesenchymal-to-epithelial transition (MET), to form a metastatic lesion at a new site (Heerboth et al. 2015).

As mentioned above, small RhoA GTPases regulate cellular functions such as cell invasion and metastasis formation. They are crucial in the amoeboid and mesenchymal migration (Friedl & Wolf 2003) and in the regulation of metalloproteinases (Lozano et al. 2003), which facilitate cell invasion by degrading the extracellular matrix (Stetler-Stevenson 2001). Increased RhoA activity has been connected to the transformation of mice embryo fibroblast cells (*i.e.* NIH 3T3) into an invasive fibroblastoid phenotype (Sander et al. 1999). Accordingly, expression and activity of RhoA were found to be enhanced in several human cancers (Kamai et al. 2001; Fukui et al. 2006; Horiuchi et al. 2008; Gilkes et al. 2014) and have been linked to multiple steps in tumour progression (Vega & Ridley 2008). Increased expression of RhoA was strongly related to poor prognosis in human hepatocellular carcinoma (Fukui et al. 2006; Hu et al. 2013) as well as in oesophageal squamous cell carcinoma, ovarian carcinoma and testicular germ cell tumour (Kamai et al. 2001; Faried et al. 2005; Horiuchi et al. 2008), while the suppression of RhoA signalling stimulated apoptosis of gastric cancer cells (Xu et al. 2012).

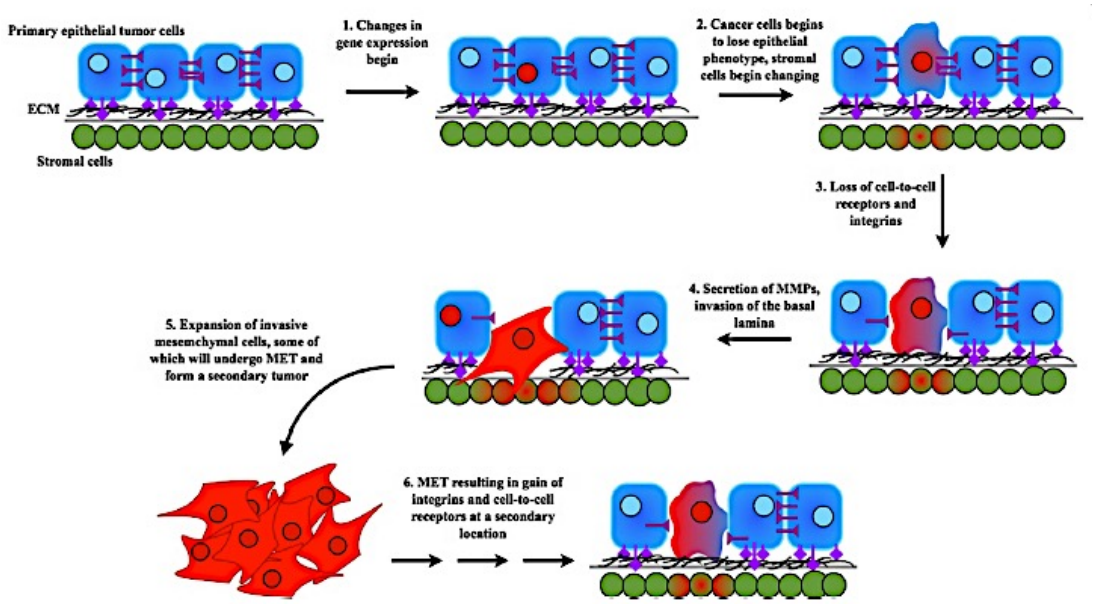


Figure 3: Type 3 epithelial-to-mesenchymal transition (EMT) during tumour dissemination. Blue = primary epithelial tumour cells; green = stromal cells; purple = cell-to-cell receptors and integrins; red/blue = cells undergoing EMT; red = invasive mesenchymal cells. ECM = extra cellular matrix; MET = mesenchymal-to-epithelial transition (Heerboth et al. 2015).

2.4.2 Regulation of NET1

There are numerous ways in which NET1 can be regulated on a transcriptional level. Dutertre and colleagues (2010) reported that oestrogens and progesterone were able to increase NET1 transcription in breast cancer cells. In pigmented epithelial cells and human keratinocytes, transforming growth factor beta (TGF-β) stimulates NET1 by small mother against decapentaplegic (Smad) 2/3 proteins and extracellular signal-regulated kinases 1/2 -dependent pathways, respectively (Shen et al. 2001; Lee et al. 2010; Papadimitriou et al. 2011). Other studies have reported an increase in NET1 expression levels mediated by IL-2 in human lymphocytes (Mzali et al. 2005) and by tumour necrosis factor alpha together with lysophosphatidic acid (LPA) in gastric

cancer cells (Leyden et al. 2006; Murray et al. 2008). Gene expression-regulating non-messenger single-strand RNAs or micro-RNAs (miRs) have been associated with NET1 regulation as well (Ambros 2004). In particular, miR-22 regulates NET1 expression in leukaemia cells, while miR-24 decreases NET1 mRNA levels in human keratinocytes (Papadimitriou et al. 2011; Ahmad et al. 2014).

Although sub-cellular localization, post-translational modification and degradation are considered the main regulators of proteins encoded by NET1, many specific stimuli and molecular mechanisms are still unknown. Another possible means of regulation is so-called nuclear sequestration. The NET1 protein is activated when it is relocated from the nucleus to the cytoplasm (Schmidt & Hall 2002). More recent studies have found that this relocation can be stimulated by epidermal growth factor and Ras-related C3 botulinum toxin substrate 1 (Rac1) proteins (Carr et al. 2013a; Song et al. 2015; Ulu & Frost 2016), while LPA leads to cytoplasmic accumulation (Song et al. 2015).

2.4.3 NET1, EMT and cancer progression

There are numerous studies investigating the role of NET1 in cancer progression. The NET1 protein promotes EMT through various mechanisms, *e.g.* it is critical for TGF-β1-induced cytoskeletal reorganization and N-cadherin expression (Figure 4; from Lee et al. 2010) and is involved in the regulation of cell-to-cell contacts (Carr et al. 2009). The NET1 protein was found to be overexpressed in human gastric adenocarcinoma, hepatocellular carcinoma and breast adenocarcinoma, and its knockdown decreased cell invasion and migration (Leyden et al. 2006; Murray et al. 2008; Shen et al.

2008; Carr et al. 2013b). An increased NET1 expression was noted in oesophageal cancer, and its high expression was an independent prognostic factor for lymph node metastasis (Lahiff et al. 2013). Similarly, NET1 has been proposed as a prognostic marker and a potential therapeutic target for non-small cell lung cancer due to its strong association with lymph node metastasis, distant metastasis and differentiation degrees (Fang et al. 2015). In patients with gliomas, a higher NET1 protein level correlated with higher tumour grades and lower median survival (Tu et al. 2010). Finally, dual silencing of NET1 and survivin effectively inhibited proliferation in skin squamous cell carcinoma (Ji et al. 2015).

In vitro studies have shown that expression of NET1 in human tumour cells can be influenced by drugs used in the perioperative period. In particular, Ecimovic and co-workers (2014) showed that human breast cancer cells cultured under propofol exposure significantly decreased NET1 expression, reducing cell migration, a phenomenon that was reversed after NET1 silencing.

Human breast cancers and canine mammary tumours have several common characteristics (Yamagami et al. 1996; Chia et al. 2005; Morris et al. 2009; Queiroga et al. 2011; Ranieri et al. 2013; Alvarez 2014; Liu et al. 2014; Telli & Sledge 2015), which recently brought to the recognition of canine mammary tumours as a translational model of naturally occurring breast cancer in people (Alvarez 2014). However, there is a lack of studies investigating the expression of the NET1 gene in canine mammary tumour cells. Based on these considerations, the authors considered relevant to

explore the expression of NET1 gene in canine mammary tumour cells exposed to propofol and sevoflurane.

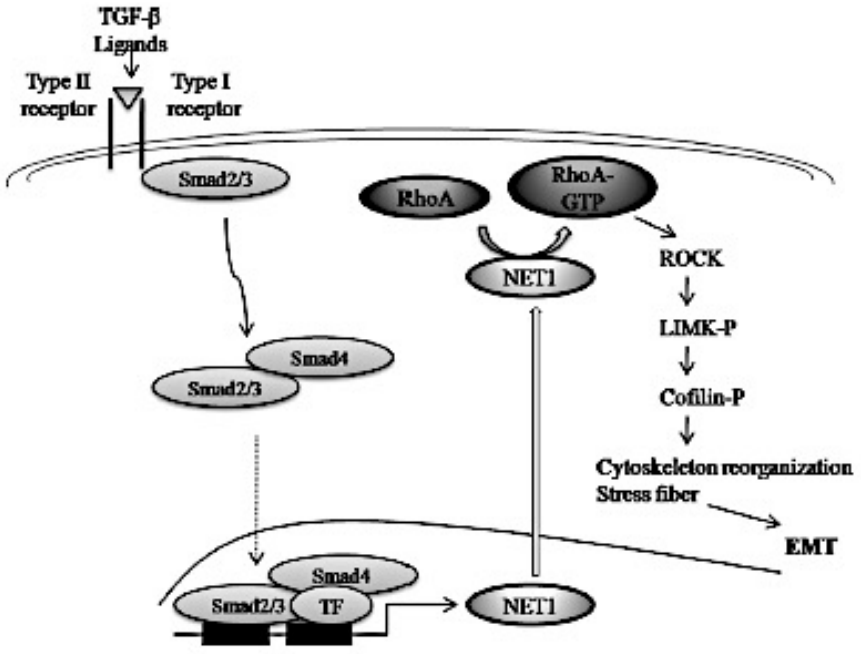


Figure 4: Mechanism of neuroepithelial transforming 1 (NET1) gene-dependent transforming growth factor (TGF)-β-induced epithelial-to-mesenchymal transition in retinal pigment epithelia cells. Stimulation of TGF-β leads to cytoskeleton reorganization via NET1-induced Ras homologue gene family, member A (RhoA) activation. GTP = guanosine triphosphate; LIMK-P = LIM protein kinase phosphorylated; ROCK = Rho-associated protein kinase; Smad = small mother against decapentaplegic protein; TF = transcription factor (Lee et al. 2010).

2.5 Rationale

Regardless of the introduction of new adjuvant therapies, surgical removal of primary lesions remains the treatment of choice for human breast cancer and canine mammary tumours (Singletary et al. 2003; Sorenmo 2003; Chia et al. 2005; Karayannopoulou & Lafioniatis 2016). This exposes human and canine patients affected with cancer to repeated anaesthetic events. In recent years,

it became strong the evidence that some anaesthetics are able to influence cancer and metastasis free time (Heaney and Buggy 2012, Bajwa et al. 2015, Byrne et al. 2016, Laudanski et al. 2016, Sekandarzad et al. 2017). Propofol and sevoflurane represent the most commonly used drugs in studies evaluating the effects of general anaesthetics on cancer progression in humans (Siddiqui et al. 2005; Wada et al. 2007; Deegan et al. 2009; Ecimovic et al. 2013; Song et al. 2014; Ye et al. 2014; Wigmore et al. 2016). However, studies evaluating the effects of these anaesthetics on cancer progression in veterinary patients are lacking. Following these considerations, the authors decided to investigate the effects of propofol and sevoflurane on canine mammary cancer cells.

Several techniques are described to asses metastatic potential of cancer cells *in vitro* (Korch et al. 2012; Borowicz et al. 2014; Yang et al. 2014). Nevertheless, the study of tumour cell proliferation has been advocated as a central element for the judgment of cancer malignant behaviour (Cooper 2000; Adan et al. 2016; Präbst et al. 2017). Being, in veterinary medicine, the study of the effects of anaesthetics on cancer recurrence at its arbour, the authors choose the MTT proliferation test as a preliminary assay for the investigation of cancer cell behaviour.

The NET1 gene has been associated with increased invasion and migration of tumour cells in human medicine (Kamai et al. 2001; Fukui et al. 2006; Horiuchi et al. 2008; Glicrease et al. 2009; Gilkes et al. 2014). Particularly, its expression has been studied in human breast cancer cells where the exposure to propofol decreased NET1 expression significantly (Ecimovic et al. 2014). To the author's knowledge, the expression of NET1 gene in canine mammary

cell lines have not been previously investigated. Considering the growing interest in human medicine for the study of genes associate with cancer development, we decided to investigate the expression of NET1 gene in canine mammary tumour cells.

Several similarities between canine and human mammary cancers brought to the recognition of canine patients as potentially suitable natural models for translational medicine (Queiroga et al. 2011, Alvarez 2014). Therefore, new findings in this research field could help optimize perioperative planning for oncologic surgery in both veterinary and human patients. Moreover, new insights could encourage investigations into propofol potential as an anti-cancer therapeutic agent.

3. AIMS OF THE STUDY

This project was designed to evaluate *in vitro* the effects of clinically available formulations of propofol and sevoflurane on cell proliferation and expression of the NET1 gene in two different canine mammary tumour cell lines.

Detailed aims were as follows:

1. To investigate the effects of propofol exposure on canine mammary tumour cell proliferation rate (Study I).
2. To investigate the effects of propofol exposure on canine mammary tumour cell NET1 expression (Study II).
3. To investigate the effects of sevoflurane exposure on canine mammary tumour cell proliferation rate (Study III).
4. To investigate the effects of sevoflurane exposure on canine mammary tumour cell NET1 expression (Study III).

4. MATERIALS AND METHODS

4.1 Cell culture

All three studies presented here were performed at the Laboratory of Pathology of the Department of Veterinary Sciences - Surgery Unit, at the Veterinary Faculty of the University of Turin (Italy). We utilized two cell lines established by Uyama and colleagues in Japan (2006). This pair of canine mammary tubular adenocarcinoma cell lines derives from a 10-year-old intact female Shih Tzu dog and was established from tissues obtained after surgical intervention. The primary cell line (CIPp) originated from the primary mass of a tubular adenocarcinoma, and the metastatic cell line (CIPm) originated from tubular and solid material from a metastatic regional lymph node (Uyama et al. 2006). Cells were first grown in Roswell Park Memorial Institute medium supplemented with 10% foetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 μg mL^{-1} penicillin (Sigma-Aldrich, St. Louis, MO, USA), 100 μg mL^{-1} streptomycin (Sigma-Aldrich, St. Louis, MO, USA), 1.5 mg mL^{-1} amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 24 hours at 37°C in a humidified atmosphere with 5% carbon dioxide.

4.2 Drug exposure

Drug exposure, in terms of concentrations and times during each study, is summarized in Figure 5.

4.2.1 Propofol

In Studies I and II, cells were exposed to different concentrations of a clinically accessible propofol preparation including a lipid-based emulsion (soybean oil, 100 mg mL^{-1}; glycerol, 22.5 mg mL^{-1}; egg lecithin, 12 mg mL^{-1}) and sodium hydroxide adjuvant to regulate the pH (LE propofol; 10 mg mL^{-1}; Vetofol, Esteve SpA, Milan, Italy).

In Study I, LE propofol was diluted with cell medium to 1, 5 or 10 µg mL^{-1} (P1, P5 and P10 treatments, respectively) prior to incubating the cells. Medium containing LE propofol was subsequently added to the cell cultures for 6 and 12 hours. The experiment was performed six times for each condition, and cells grown in culture medium alone were used as a control (C).

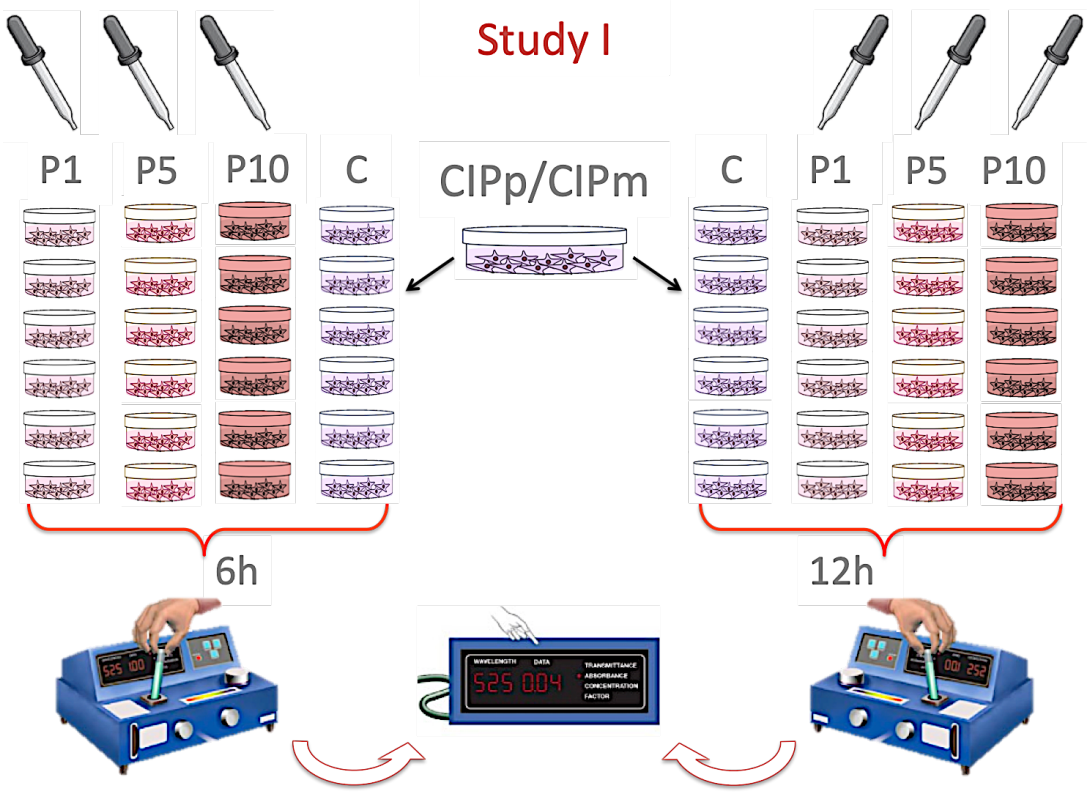
In Study II, cells were treated with LE propofol diluted with cell medium to 1 and 10 µg mL^{-1} (P1 and P10 treatments, respectively) for 6, 12, 24 and 48 hours. The experiment was performed in triplicate, and cells cultured without anaesthetics were used as a baseline condition (B).

The chosen concentrations correlated with described blood levels of propofol in greyhounds (Hughes & Nolan 1999) after the provision of clinically applicable doses intravenously (*i.e.* 4 mg kg^{-1} followed by infusions of 0.2 - 0.4 mg kg^{-1} min^{-1}).

4.2.2 Sevoflurane

In Study III, a clinically available sevoflurane formulation (Sevorane, AbbVie Oy, Espoo, Finland) was utilized for the treatments at three different concentrations: 1, 2.5 or 4 mM (S1, S2.5 and S4 treatments, respectively).

Cells grown only in the culture medium were used as a control. Medium containing sevoflurane (S1, S2.5 and S4 concentrations) was added every hour to the cell culture to avoid decreases in drug concentration over time due to evaporation, as suggested by Ecimovic and colleagues (2013). The sevoflurane concentrations were chosen since they were previously used in a study in human medicine where sevoflurane was applied on breast cancer cells to investigate changes in cell proliferation, migration and invasion (Ecimovic et al. 2013).



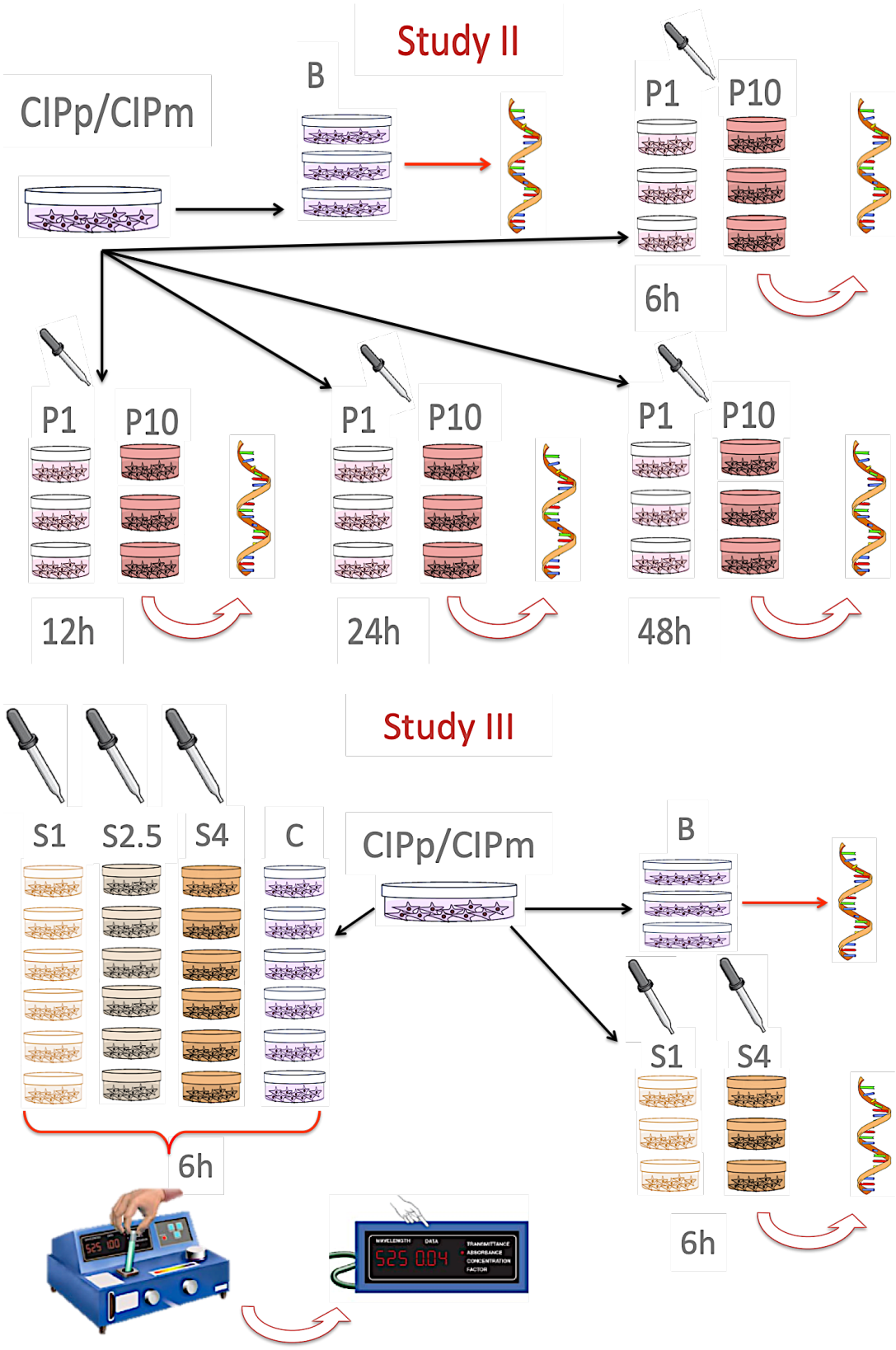


Figure 5: Summary of study designs. In Study I, primary (CIPp) and metastatic (CIPm) canine mammary tubular adenocarcinoma cells were exposed to different concentrations of a

lipid-based propofol emulsion (P1: 1 μg mL^{-1}; P5: 5 μg mL^{-1}; P10: 10 μg mL^{-1}) or cultured in a control cell culture medium (C). After 6 and 12 hours of exposure, absorbance was compared between treated cells and controls. In Study II, CIPp and CIPm were exposed to P1 and P10 or cultured in a baseline cell culture medium (B). After 6, 12, 24 and 48 hours of exposure, cells were lysed and the concentration of neuroepithelial transforming gene 1 (NET1) was compared between treated cells and baseline. In Study III, CIPp and CIPm cells were exposed to different concentrations of a commercially available sevoflurane formulation (S1: 1 mM; S2.5: 2.5 mM; S4: 4 mM) or cultured in a control cell culture medium. After 6 hours of exposure, absorbance was compared between treated cells and controls. Subsequently, CIPp and CIPm cells were exposed to S1 and S4 or cultured in a baseline cell culture medium, and after 6 hours of exposure cells were lysed and the concentration of NET1 was compared between treated cells and baseline.

4.3 Proliferation assay

In Studies I and III, cell proliferation after the exposure to propofol and sevoflurane was evaluated in both cell lines with the use of a MTT colorimetric assay for cell survival and proliferation (MTT, Sigma Aldrich, St. Louis, MO, USA), performed according to Tada and co-authors (1986). This technique quantifies the conversion of MTT into purple formazan crystals, which are produced by redox activity of living cells. A lessening of cellular redox activity implies a decrease in cell viability (Van Meerloo et al. 2011).

Previously, cells were cultured in a 75 cm^2 standard tissue culture flask (Sarstedt Ltd., Dublin, Ireland) as monolayers. Media were changed every 3 days. Before each experiment, cells were harvested from 70% confluent cultures by trypsinization and quantified with an automated cell counter (Automated Cell Counter TC20, Bio-Rad, Milan, Italy).

The time-dependent exponential cell growth curve was preliminarily evaluated at 4, 6 and 12 hours for each cell line to define ideal experimental settings. Cells were seeded into a normal cell culture medium in a range from 1000 to 10 000 cells per well. The trial was performed in a 96-well plate in triplicate (Eppendorf Cell Culture Plate, Eppendorf S.r.l., Milan, Italy) and a proliferation index was obtained with the MTT colorimetric assay (see below). On the basis of the resulting time-dependent exponential cell growth curve, a concentration of 3000 cells per well was found to be the most appropriate to show time-related cell growth (*e.g.* no indications of cell death consequent to over-confluence) and was therefore selected for assessing cell proliferation during 6 and 12 hours of exposure to treatments. Thus, cells were seeded at a density of 3000 cells per well for a total of 6 experimental wells in a 96-well cell culture plate (Eppendorf Cell Culture Plate, Eppendorf S.r.l., Milan, Italy) and incubated with 100 µL of normal cell culture medium for 12 hours to allow homogeneous cell adhesion.

Once the different exposure times were reached, the solutions containing treatments with propofol or sevoflurane were removed. Afterwards, 20 µL of MTT diluted in phosphate-buffered saline at a 5 mg mL^{-1} concentration and a 7.5 pH was supplemented to every well and incubated for 4 hours at 37°C. Following the initial incubation period, 0.1 mL of 10% sodium dodecyl sulphate (Sigma-Aldrich, St. Louis, MO, USA) diluted in a solution of 0.01 M hydrochloric acid was supplemented to every well to dissolve the formazan crystals and incubated overnight. Subsequently, quantifications were obtained with a spectrophotometer (Microplate Model 680, Bio-Rad, Milan, Italy) on an ELISA plate reader at 590 nm. A total value of absorbance was obtained.

Absorbance values inferior to those of controls indicated a decrease in cell proliferation rate, while values higher than those of controls indicated an increase in cell proliferation rate (Van Meerloo et al. 2011).

4.4 Quantitative PCR

In Studies II and III, a qPCR was performed to evaluate the expression of NET1 after treatment exposure.

Three hundred thousand cells were seeded in triplicate in 6-well culture plates (Eppendorf Cell Culture Plate, Eppendorf S.r.l., Milan, Italy). After about 6 hours of incubation to permit cell attachment, they were exposed to the aforementioned concentrations of propofol and sevoflurane for 6 and 12 hours in the case of propofol, and for 6 hours only in the case of sevoflurane. In Study III, as in the protocol for the MTT assay, the same concentrations of sevoflurane were added to the cell cultures every hour to compensate for the evaporation tendency of this agent. Cells cultured without treatment were used as controls.

To isolate total ribonucleic acid (RNA), culture media were removed from the cell cultures at the end of the treatment, and 0.5 mL of a ready-to-use reagent designed to isolate high-quality total RNA (TRIzol, Sigma-Aldrich, Dublin, Ireland) was added to each well to lyse the cells, according to the manufacturer's instructions. Once a microscopic examination revealed cells to be lysed, the cell lysate was transferred to a clean 1.5 mL microfuge tube. Thereafter, 200 µL of chloroform was added. The mixture was gently shaken, left at room temperature (25°C) for 15 minutes and centrifuged at 13 000 *g* for

15 minutes at 4°C. The upper aqueous layer was transposed into another clean 1.5 mL tube carefully without touching the genomic and protein-containing interphase. A total of 0.5 mL of ice-cold isopropanol was added to the aqueous phase, the tube was gently shaken and left to stand on ice for 10 minutes before being centrifuged at 13 000 *g* for another 10 minutes at 4°C. The supernatant was removed and 1 mL of sterile ethanol (75%) was added in order to wash the pellet by gently centrifuging (7500 *g* for 5 minutes). After ethanol removal, the pellet was allowed to air dry for 5 minutes before being re-suspended in 50 µL of nuclease-free water by heating it at 60°C for 15 minutes. Total RNA was quantified with an automated electrophoresis system (Experion Electrophoresis System, Bio-Rad, Milan, Italy), and cDNA was synthesized from 1 µg of total RNA using a reverse transcription kit (QuantiTect Reverse Transcription kit, Qiagen, Milan, Italy). According to the manufacturer's instructions, 1 µg of total RNA was incubated with 2 µL of deoxyribonuclease buffer (gDNA Wipeout Buffer, Qiagen, Milan, Italy) and ribonuclease-free water to reach a total volume of 14 µL for 2 minutes at 42°C and then left for 10 minutes on ice. Thereafter, 1 µL of reverse transcriptase (Quantiscript Reverse Transcriptase, Qiagen, Milan, Italy), 4 µL of a dedicated buffer (Quantiscript RT Buffer 5X, Qiagen, Milan, Italy) and 1 µL of a dedicated primer mix (RT Primer mix, Qiagen, Milan, Italy) were added and incubated for 15 minutes at 42°C, followed by 3 minutes at 95°C to inactivate the reverse transcriptase. To evaluate the relative amount of specific NET1 transcript, 1 µL of cDNA obtained as previously described was used for qPCR. Briefly, 1 µL of cDNA was subjected to qPCR with a dedicated detection chemistry system (IQ SYBR Green Supermix, Bio-Rad, Milan, Italy) and an optical

software system (IQ5 Optical System Software, Bio-Rad, Milan, Italy). Primer sequences are listed in Table 5 (Argano et al. 2017).

The reaction conditions were reverse transcription for 3 minutes at 95°C (1 cycle), followed by denaturation for 30 seconds at 95°C, and annealing for 30 seconds at 60°C (35 cycles). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels were used to normalize NET1 expression.

Values for delta cycle threshold (ΔCt) were obtained in triplicate for each sample. Gene expression was calculated using a relative quantification assay corresponding to the comparative Ct method: the amount of target gene, identified through its cycle threshold, was normalized by subtracting the cycle threshold of the endogenous housekeeping gene (GAPDH). These sample ΔCt values were related to the appropriate controls with linear models (see below).

Table 5: Primer sequences used in quantitative PCR (Argano et al. 2017).

Primer	Gene	Sequence
Forward	NET1	5’-CATCAAGAGGACGATCCGGG-3’
Reverse	NET1	5’-ATTGCTTGGCTCCTCTTGCT-3’
Forward	GAPDH	5'-GGCACAGTCAAGGCTGAGAAC-3'
Reverse	GAPDH	5'-CCAGCATCACCCCATTTGAT-3'

4.5 Statistical analysis

In Study I, the mean absorbance value from controls was compared with mean absorbance values from treatments using a 3-way ANalysis Of VAriance

(ANOVA) with time, cell line and concentration of LE propofol as factors. Normality of residuals, which is an assumption of linear models, was tested with a Shapiro-Wilks test of normality of residuals. Approximate equality of variances within groups was checked by visual inspection. Data obtained with the MTT assays are presented as mean ± standard error and ranges for the percentage increases or decreases of cell proliferation rates.

In Studies II and III, one overall mean value of ΔCt was used for statistical analysis per biological sample (*i.e*. mean values of the technical triplicates). Amplification with qPCR is inherently an exponential process, such that the error structure is approximately linear in Ct values (and linear in the log of the inferred number of molecules). Hence, an analysis with linear models is indicated (Pfaffl 2001).

In Study II, ΔCts were compared using a 3-way ANOVA test (time, cell line, concentration of LE propofol as factors) or a Student T-test as relevant. Data are presented as mean ΔCt values over time for both cell lines.

In Study III, a 2-way ANOVA test was performed for the evaluation of cell proliferation using as target variables the mean values of absorbance at 6 hours of treatment, while treatments (the three sevoflurane concentrations and the control) and cell types (primary and metastatic cells) were used as explanatory variables. Similarly to Study I, data obtained with the MTT assays are presented as mean ± standard error and ranges for the percentage increases or decreases of cell proliferation rates.

One-way ANOVA tests were used in Study III to analyse the expression of NET1; the target variables were mRNA expressions of NET1 and the

treatments (two sevoflurane concentrations, S1 and S4, and the control) were the explanatory variables.

In all sets of ANOVA tests, pairwise differences between treatments and controls were tested for significance. A value of $p < \alpha = 0.05$ was considered statistically significant. Tests for the correction for multiple comparisons were not performed.

All statistical analyses were performed with an open-source statistical software package (R-studio, version 3.2.0, Boston, MA, USA; www.r-project.org).

5. RESULTS

5.1 Cell proliferation under propofol treatments (I)

A Shapiro-Wilks test of the residuals indicated no deviation from normality.

After 6 hours of exposure, no significant differences were found in CIPp between the mean absorbance value of the P1 treatment group and that of control cells. Treatments P5 and P10 showed a significant increase ($p = 0.0137$ and $p = 0.0005$) in cell proliferation rate of 18% and 27%, respectively (Figure 6 A; from Argano et al. 2019a). In CIPm cells at the same incubation time, no significant changes were noticed in cell proliferation between control and any LE propofol-treated cell (Figure 6 B; from Argano et al. 2019a).

After 12 hours of exposure, all LE propofol concentrations produced a significant increase in cell proliferation in CIPp cells (Figure 7 A; P1 = 105% $p = 7.48e-07$; P5 = 114% $p = 2.32e-07$; P10 = 123% $p = 6.94e-08$; from Argano et al. 2019a) and a significant decrease in cell proliferation in CIPm cells (Figure 7 B; P1 = -22% $p = 0.0001$; P5 = -14% $p = 0.0075$; P10 = -22% $p = 0.0002$; from Argano et al. 2019a).

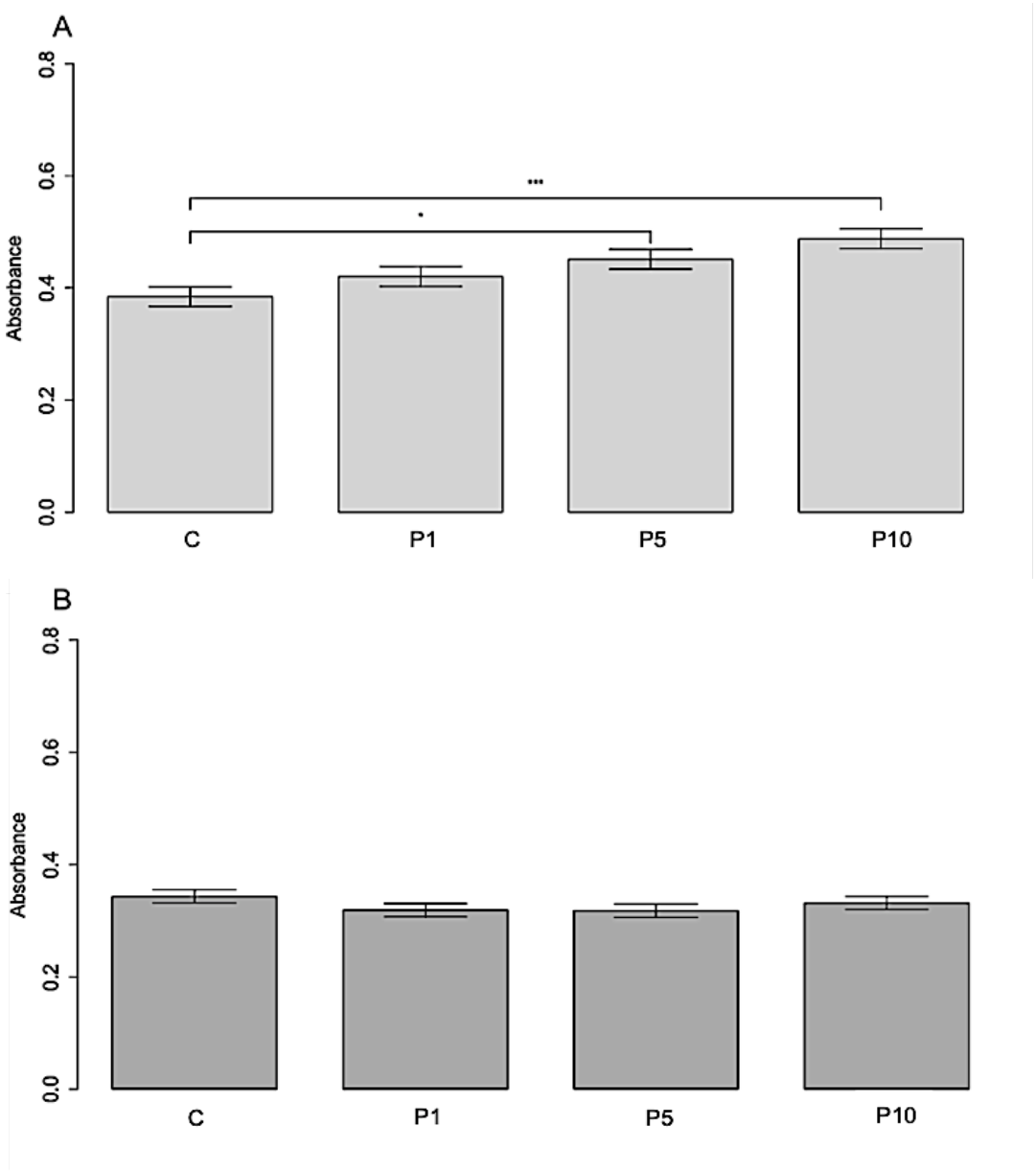


Figure 6: Changes in absorbance values between primary (A; CIPp) and metastatic (B; CIPm) canine mammary tubular adenocarcinoma cells exposed to different concentrations (P1: 1 µg mL^{-1}; P5: 5 µg mL^{-1}; P10: 10 µg mL^{-1}) of a lipid-based propofol emulsion compared with control cells (C) after 6 hours of drug exposure. Significantly different comparisons are indicated by asterisks (**: $p < 0.01$; *: $p < 0.05$; from Argano et al. 2019a).

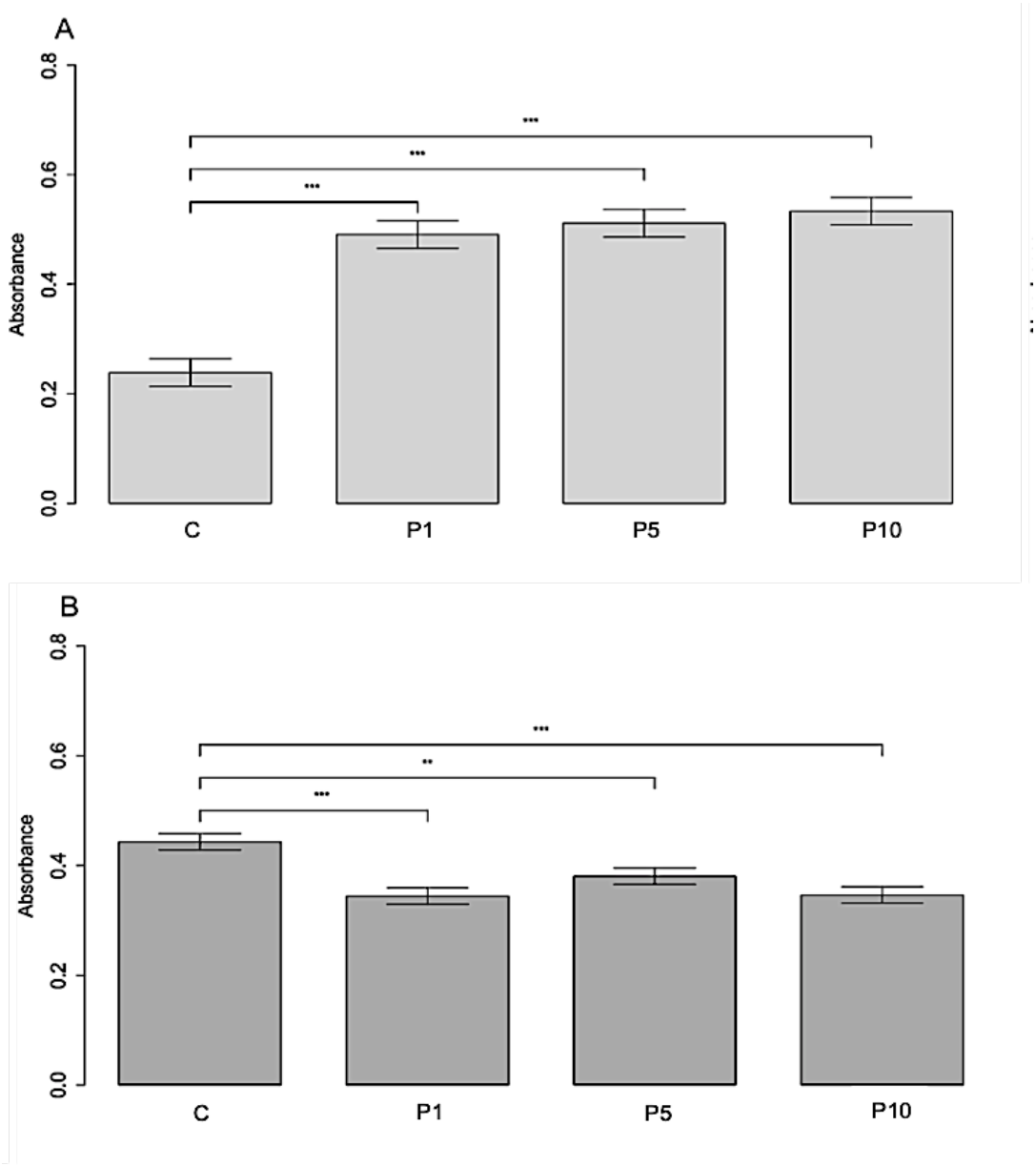


Figure 7: Changes in absorbance values between primary (A; CIPp) and metastatic (B; CIPm) canine mammary tubular adenocarcinoma cells exposed to different concentrations (P1: 1 µg mL^{-1}; P5: 5 µg mL^{-1}; P10: 10 µg mL^{-1}) of a lipid-based propofol emulsion compared with control cells (C) after 12 hours of drug exposure. Significantly different comparisons are indicated by asterisks (***: $p < 0.001$; **: $p < 0.01$; from Argano et al. 2019a).

5.2 NET1 expression under propofol treatments (II)

Expression of NET1 gene was detected in both primary and metastatic cell lines cultured without propofol in the sole culture medium. A significantly higher NET1 expression was observed in CIPp compared to CIPm without drug exposure (median ΔCt 5.82 and 6.48, respectively; p = 0.0004).

Tables 6 and 7 report the mean ΔCt values observed over time for CIPp and CIPm, respectively, after exposure to P1 and P10 (Argano et al. 2017).

Table 6: Neuroepithelial transforming gene 1 (NET1) expression reported as mean delta cycle threshold (ΔCt) values in the primary canine tubular adenocarcinoma cells (CIPp) after 6, 12, 24 and 48 hours of exposure to P1 (A; 1 μg mL^{-1}) or P10 (B; 10 μg mL^{-1}; from Argano et al. 2017).

A	CIPp		B	CIPp	
	P1			P10	
	6 hours			6 hours	
	Mean ΔCt	5.11		Mean ΔCt	4.93
	12 hours			12 hours	
	Mean ΔCt	6.70		Mean ΔCt	6.88
	24 hours			24 hours	
	Mean ΔCt	7.10		Mean ΔCt	6.80
	48 hours			48 hours	
	Mean ΔCt	6.35		Mean ΔCt	4.91

Table 7: Neuroepithelial transforming gene 1 (NET1) expression reported as mean delta cycle threshold (ΔCt) values in the metastatic canine tubular adenocarcinoma cells (CIPm) after 6, 12, 24 and 48 hours of exposure to P1 (A; 1 µg mL^{-1}) or P10 (B; 10 µg mL^{-1}; from Argano et al. 2017).

A	CIPm		B	CIPm	
	P1			P10	
	6 hours			6 hours	
	Mean ΔCt	6.81		Mean ΔCt	7.35
	12 hours			12 hours	
	Mean ΔCt	7.00		Mean ΔCt	7.04
	24 hours			24 hours	
	Mean ΔCt	6.67		Mean ΔCt	6.81
	48 hours			48 hours	
	Mean ΔCt	6.75		Mean ΔCt	6.44

After 6 hours of exposure, CIPp cells treated with both concentrations of LE propofol showed a significantly higher (P1 p = 0.0373; P10 p = 0.0104) NET1 expression than at baseline (Figure 8; from Argano et al. 2017). In CIPm cells, a significant difference (p = 0.0202) was found between baseline and the higher concentration of LE propofol, the latter showing lower gene expression (Figure 9; from Argano et al. 2017). After 12 hours of exposure, in CIPp cells NET1 mRNA levels were significantly decreased with both concentrations of LE propofol (P1 p = 0.0004; P10 p = 4.80e-06; Figure 8). In CIPm cells, NET1 expression was significantly decreased (p = 0.0261) with the higher concentration of LE propofol compared with baseline (Figure 9). After 24 hours of exposure, in CIPp cells both concentrations of LE propofol induced a significant reduction (P1 p = 2.67e-05; P10 p = 0.0007) in NET1 expression

compared with baseline (Figure 8). No significant differences were found at 24 hours in CIPm cells. After 48 hours of exposure, in CIPp cells the higher concentration of LE propofol induced a significant increase ($p = 0.0361$) in gene expression compared with baseline (Figure 8). No significant differences were found at 48 hours in CIPm cells.

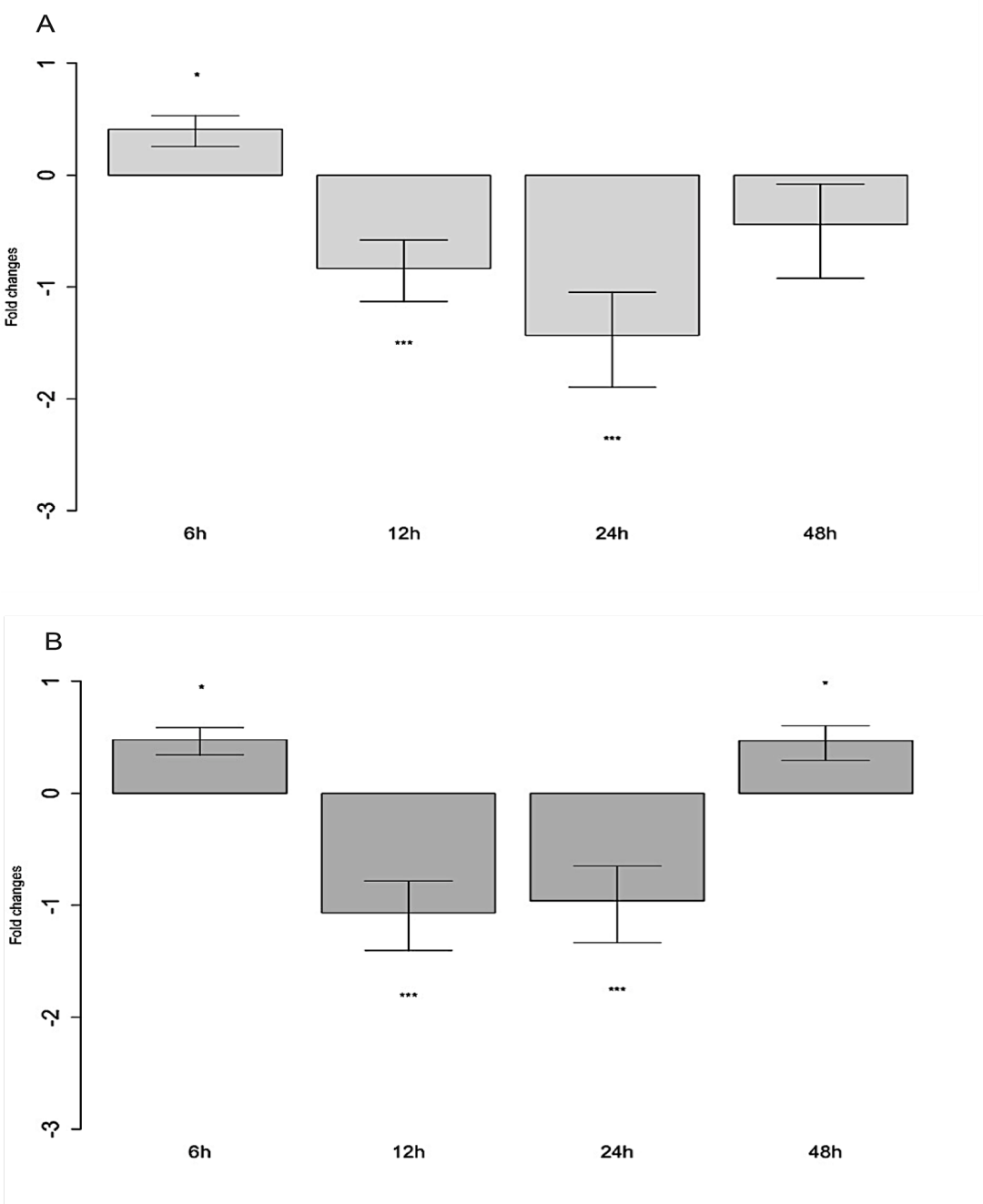


Figure 8: Means and standard errors of fold changes (ΔCt) over time at 6, 12, 24 and 48 hours (h) of exposure in neuroepithelial transforming gene 1 (NET1) expression in primary canine tubular adenocarcinoma cells (CIPp) exposed to different concentrations of a lipid-based propofol emulsion (A; P1 = 1 µg mL^{-1} and B; P10 = 10 µg mL^{-1}) *versus* baseline. Significantly different comparisons to the baseline are indicated by asterisks (* = $p < 0.05$, *** = $p < 0.005$; from Argano et al. 2017).

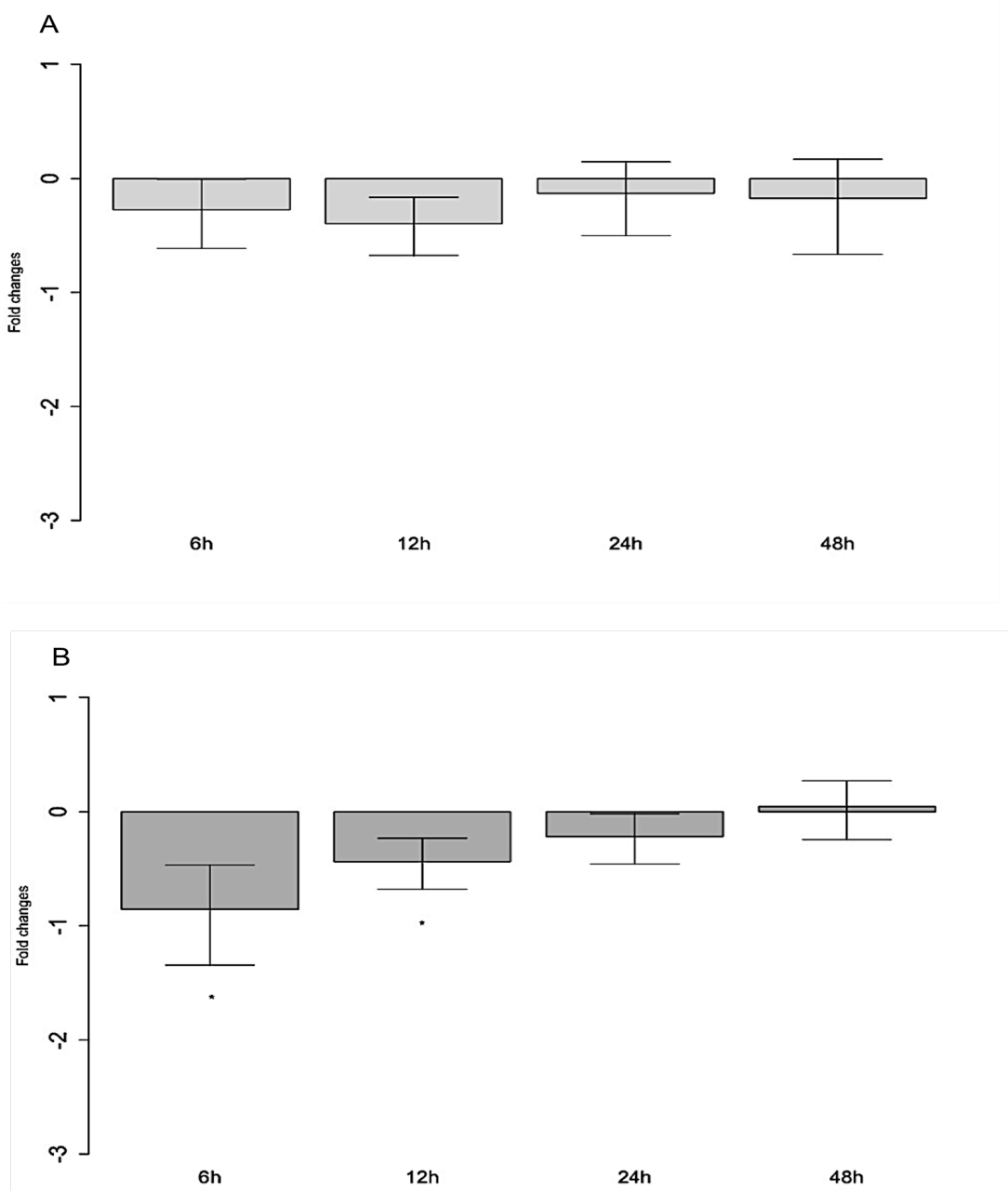


Figure 9: Means and standard errors of fold (ΔCt) over time at 6, 12, 24 and 48 hours (h) of exposure in neuroepithelial transforming gene 1 (NET1) expression in metastatic canine tubular adenocarcinoma cells (CIPm) exposed to different concentrations of a lipid-based propofol emulsion (A; P1 = 1 µg mL^{-1} and B; P10 = 10 µg mL^{-1}) *versus* baseline. Significantly different comparisons to the baseline are indicated by asterisks (* = $p < 0.05$; from Argano et al. 2017).

5.3 Cell proliferation under sevoflurane treatments (III)

Normal distribution of residuals was confirmed by a Shapiro-Wilks test.

A significant increase in cell proliferation rate relative to controls was observed in CIPp treated with S1 and S2.5 (Figure 10 A; 23% p = 0.0519 and 13% p = 0.0083, respectively; from Argano et al. 2019b). Conversely, a significant decrease in cell proliferation rate was found in CIPm treated with all of the tested concentrations of sevoflurane (Figure 10 B; S1 = -33% p = 4.38e-06; S2.5 = -41% p = 2.36e-07; S4 = -62% p = 2.59e-10; from Argano et al. 2019b).

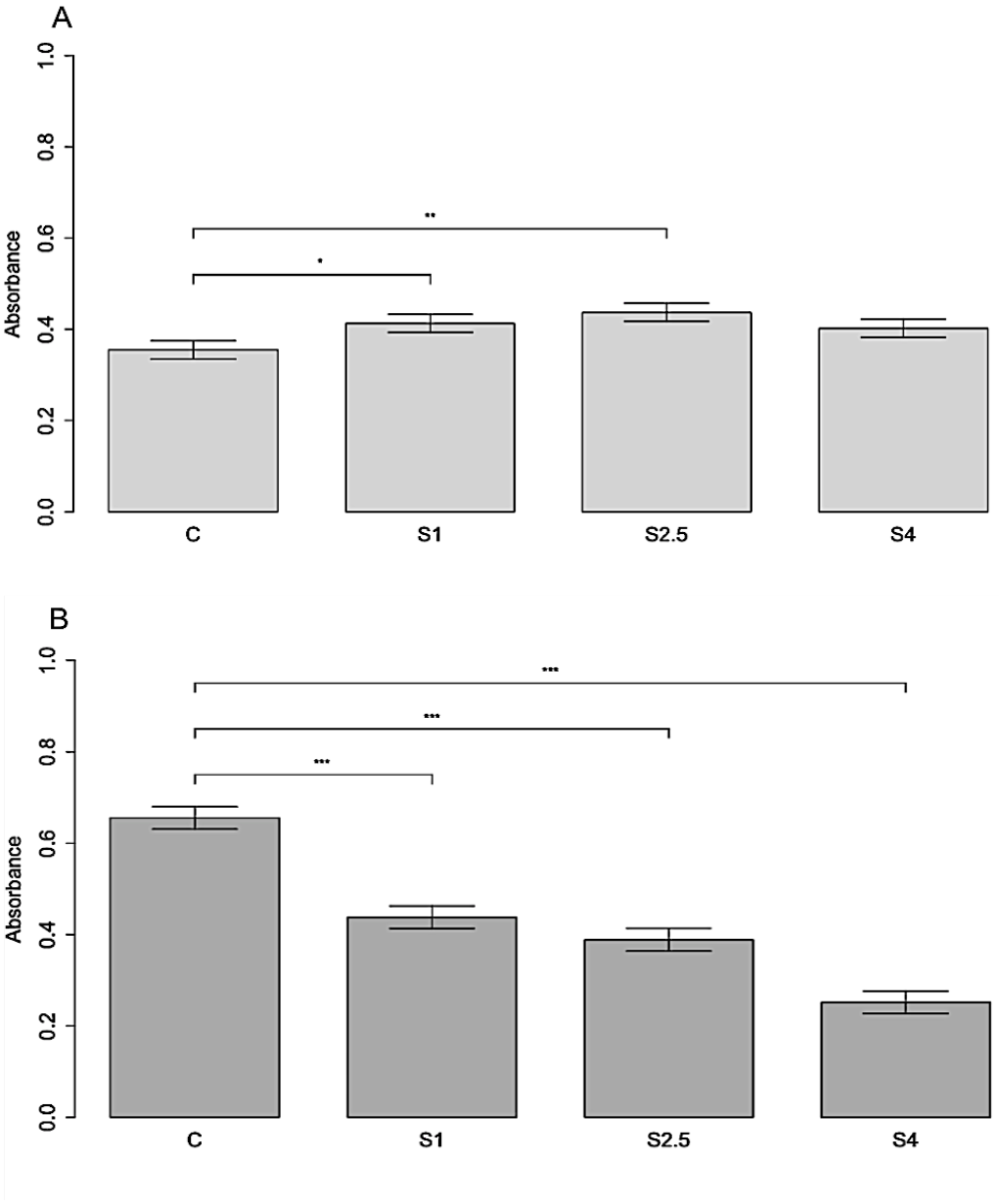


Figure 10: Means and standard errors of absorbance values between primary (A; CIPp) and metastatic (B; CIPm) canine mammary tubular adenocarcinoma cells exposed to different concentrations (S1: 1 mM; S2.5: 2.5 mM; S4: 4 mM) of a commercially available sevoflurane formulation compared with control cells (C). Significantly different comparisons are indicated by asterisks (***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; from Argano et al. 2019b).

5.4 NET1 expression under sevoflurane treatments (III)

Neither sevoflurane concentration (S1 or S4) induced a significant change in NET1 expression in CIPp cells compared with baseline (Figure 11 A; from Argano et al. 2019b). A significant increase ($p = 0.0468$) in gene expression was observed only in CIPm between cells treated with S4 and baseline (Figure 11 B; from Argano et al. 2019b).

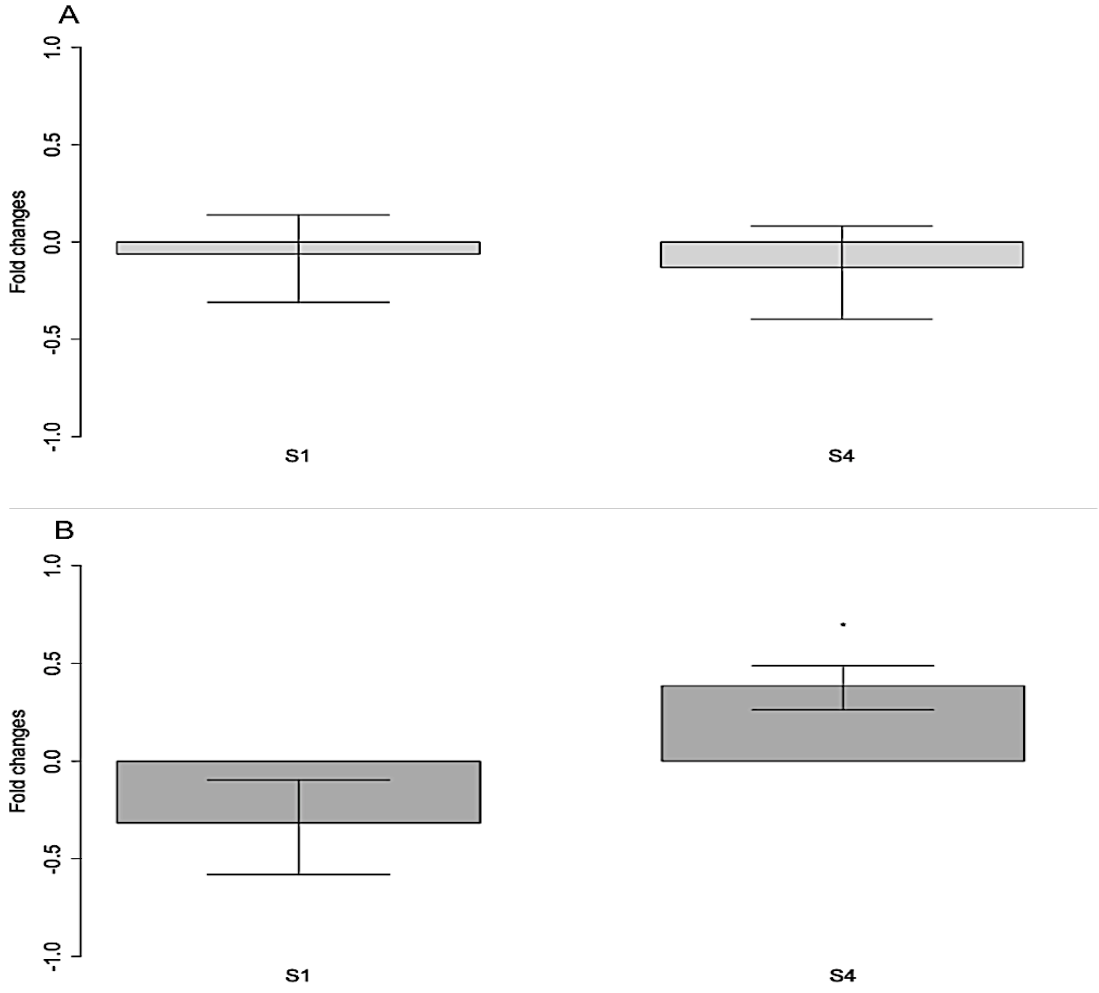


Figure 11: Means and standard errors of fold changes (ΔCt) in neuroepithelial transforming gene 1 (NET1) expression in primary (A; CIPp) and metastatic (B; CIPm) canine mammary tubular adenocarcinoma cells exposed to different concentrations (S1: 1 mM; S4: 4 mM) of a commercially available sevoflurane formulation. Significantly different comparisons are indicated by asterisks (*: $p < 0.05$; from Argano et al. 2019b).

6. DISCUSSION

In the present study we demonstrated that the highest, but still clinically applicable, concentrations tested of LE propofol and the longest of the exposure times chosen effectively changed cell proliferation. In detail, they resulted in an increase in cell proliferation rate when applied to primary canine tubular adenocarcinoma cells and in a decrease in cell proliferation when applied to metastatic tubular adenocarcinoma cells. Lipid-based propofol emulsion treatments effectively modified NET1 expression of canine mammary cancer cells. Both of the concentrations examined here induced a decrease in gene expression at most of the treatment time points, although increases in gene expression were also observed. Sevoflurane treatments modified cell proliferation rate in both cell lines, showing an increase or a decrease in cell proliferation when applied on CIPp or CIPm cells, respectively, compared with cells grown in the sole cell culture medium. The expression of NET1 increased only after the treatment with sevoflurane 4 mM in metastatic cells.

6.1 Cell proliferation under propofol treatments (I)

Lipid-based propofol emulsion treatments significantly modified cell proliferation rate of cultured canine tubular adenocarcinoma cells in a cell line-dependent manner. Lipid-based propofol emulsion treatments significantly augmented and reduced cell proliferation in CIPp and CIPm cells, respectively,

compared with the control group. Time effects (*i.e.* 6 hours *versus* 12 hours of exposure) were also noticed, mainly in CIPm.

Results on the effects of propofol on cancer cell growth from previous trials are controversial. For instance, propofol stimulated proliferation in gallbladder tumour cells (Zhang et al. 2012) and neuroblastoma cells (Wu et al. 2011), but decreased proliferation in osteosarcoma cells (Ye et al. 2014). Propofol has been advocated to prevent breast cancer cell proliferation *in vitro*, supporting the theory that it may reduce the incidence of cancer recurrence and prolong survival time in women with breast cancer (Siddiqui et al. 2005; Deegan et al. 2009).

In breast cancer cells, propofol at concentrations of 5 and 10 μg mL^{-1} suppressed cell growth when applied to Michigan Cancer Foundation-7 (MCF-7) oestrogen receptor-positive and on M.D. Anderson-Metastasis Breast-231 (MDA-MB-231) oestrogen receptor-negative cell lines (Ecimovic et al. 2014). Propofol conjugates were able to significantly prevent cell growth when applied to MDA-MB-231 oestrogen receptor-negative breast cancer cells (Siddiqui et al. 2005), even though these effects were observed only with concentrations of approximately 20 μg mL^{-1} of propofol conjugates. In Study I, a decrease of absorbance was observed in CIPm cell lines treated with LE propofol for 12 hours at concentrations between 1 and 10 μg mL^{-1}, which are considered to stay within the blood concentration levels achieved in dogs anaesthetized with propofol. In fact, whole blood propofol concentrations measured by Hughes and Nolan (1999) after administering 4 mg kg^{-1} of propofol followed by infusions of 0.2 - 0.4 mg kg^{-1} min^{-1} to 8 greyhounds ranged from 1.21 to 6.77 μg mL^{-1}.

Unexpectedly, in the present study, a rise in cancer cell proliferation was noticed when applying LE propofol at nearly all concentrations and at both time points on CIPp. In a recent trial, also breast cancer cells (*i.e.* MDA-MB-231) treated for 1, 4 and 12 hours with propofol (2 - 10 µg mL^{-1}) exhibited an increase in proliferation rate in a dose- and time-dependent manner (Meng et al. 2017), with results appearing to disagree with those of Siddiqui et al. (2005) and Deegan et al. (2009). Meng and co-workers (2017) hypothesized that the augmented proliferation was partially connected with inhibition of the expression of p53, a gene involved in the suppression of cell mutation. This hypothesis is noteworthy considering that human and canine mammary tumours share molecular characteristics such as variations in the expression of steroid receptors (Chia et al. 2005) and mutations of suppressor gene p53 (Morris et al. 2009).

It is not well defined why LE propofol produced dissimilar effects in CIPp and CIPm cell lines in our study. A strong cell type dependency has not been described before. For instance, in a study by Ecimovic and co-workers (2014), propofol did not lessen proliferation of breast tumour cells at any of the studied concentrations (1 - 10 µg mL^{-1}) or any of the exposure times (6, 12, 24, 36 hours) in either oestrogen receptor-positive or oestrogen receptor-negative cell lines. Therefore, an absence of propofol effect as well as an absence of dependency on dose, time and cell type were demonstrated. In Study II, a decrement in NET1 expression was detected when LE propofol was applied to CIPm. When LE propofol was applied to CIPp cells, a double effect on NET1 expression was observed: increments of gene expression were noticed at 6 and 48 hours and decrements of gene expression were noticed at 12 and 24

hours of exposure. Although the purpose of both tests (*i.e*. MTT and qPCR for NET1) was to evaluate possible propofol anti-cancer activities, the laboratory methods used were dissimilar, with one test assessing cell proliferation and the other assessing the expression of a gene linked to cell migration. Indeed, the ability to proliferate and the ability to migrate do not have to be the same in a particular cell evolutionary state or cell type and could be considerably diverse in primary and metastatic cells from the same tumour type (Martin et al. 2013).

In this study, exposure times of 6 and 12 hours were used. The choice of these exposure times was based on previously reported studies published in human medicine *in vitro* (Ecimovic et al. 2013), with the aim of facilitating comprehensive comparisons between studies.

Different tests can be selected to evaluate cell proliferation. Hence, different outcomes between research trials may be the effect of dissimilar methodologies. In the present project, the MTT proliferation assay was chosen because it is one of the most commonly used techniques for the evaluation of mammary tumour response to drug exposure. The MTT assay is based on the quantification of absorbance, which is linked to variations in cell number (Tada et al. 1986). The reason why the cell number changes (*e.g.* augmentation or reduction in proliferation, cell death or apoptosis) cannot be determined with this method. Other trials, for instance, use techniques that associate the assessment of cell growth utilizing the Water Soluble Tetrazolium-1 assay and the evaluation of cellular viability and apoptosis utilizing a Vybrant apoptosis test kit (Siddiqui et al. 2005).

6.2 NET1 expression under propofol treatments (II)

To the author's knowledge, this is the first study to focus on the biological effects of a clinically available propofol formulation on NET1 expression in canine mammary cancer cell lines. We observed that both P1 and P10 propofol concentrations induced a decrease in NET1 expression in CIPp after 12 and 24 hours of exposure. In CIPm, only the higher concentration of propofol (*i.e.* P10) caused a reduction of NET1 expression after 6 and 12 hours of exposure. Paradoxically, an increased NET1 expression was observed in CIPp with both LE propofol concentrations and with the higher LE propofol concentration after 6 and 48 hours of exposure. To the author's knowledge, this was the first time that such a divergent effect was described for a clinically available propofol formulation applied to a primary canine tumour cell line.

As already stated, the NET1 protein is crucial for TGF1-induced cytoskeletal reorganization, N-cadherin expression and RhoA activation (Garcia-Mata et al. 2007). Being a RhoA-specific GEF, the NET1 protein plays an important role in the EMT, enabling tumour cells to invade and migrate (Clark et al. 2000). Silencing this gene has been previously associated with abolition of the inhibitory effect of propofol on the migrating ability of cancer cells (Ecimovic et al. 2014). Therefore, a reduction in NET1 expression may be interpreted as a potentially anti-metastatic effect. On the other hand, an increase of NET1 expression could be interpreted as an increased ability of the tumour cells to cause metastasis (Tu et al. 2010). Consistently, the co-expression of NET1 protein and α6β4 integrin in the primary tumours of lymph

node-positive patients with invasive breast carcinoma was associated with decreased distant metastasis-free survival time (Gilcrease et al. 2009).

In the present study, we chose cell lines originating from the same individual, but with different malignant potentials to compare the effects of propofol on primary and metastatic cells. The mRNA of NET1 was detectable in both CIPp and CIPm. However, NET1 protein showed a higher expression in CIPp than in CIPm. These findings suggest that CIPp has a higher invasiveness potential than CIPm. Indeed, it has been speculated that intrinsic mechanisms promoting cell invasion and migration are enhanced in CIPp due to the fact that this cell line is the one responsible for tumour dissemination to remote locations within the body (Uyama et al. 2006). Conversely, in CIPm, mechanisms responsible for cell migration may be attenuated thereby enabling these cells to adhere to each other and grow, establishing metastatic lesions in distant organs.

The propofol doses reported in the present study were chosen to reflect the plasma concentrations obtained clinically during propofol anaesthesia in dogs (Hughes & Nolan 1999). A generic chemical form of propofol at concentrations between 1 and 10 μg mL^{-1} reduced NET1 expression by 49 - 79% in the MDA-MB-231 oestrogen receptor-negative human breast adenocarcinoma cell line and by 42 - 88% in the MCF7 oestrogen and progesterone receptor-positive human breast adenocarcinoma cell line (Ecimovic et al. 2014). In contrast to our findings where NET1 expression was reduced in CIPm only with the higher concentration of LE propofol, Ecimovic and co-authors (2014) reported a lack of dose-dependent effect in MDA-MB-231 cells and MCF7 cells. Comparably,

in Study II both doses of LE propofol influenced NET1 expression and a dose-dependent effect could not be demonstrated.

Different results were found at different time points in Study II; however, the observed time effects did not follow a linear pattern. With the shorter exposure time (*i.e.* 6 hours) an increase of NET1 mRNA levels in CIPp was observed. This finding was unexpected and appears to indicate an enhancement of malignancy in primary tumour cells when exposed to LE propofol for that period of time. However, this effect was reversed after 12 and 24 hours of exposure, with LE propofol producing a reduction in gene expression in CIPp. Thereafter, a significant increase in NET1 expression was observed once again in CIPp. A time-dependent response in the expression of NET1 in CIPp when exposed to LE propofol could be advised in the light of these observations. Some sort of time dependency was already recognised in MDA-MB-231 cells and MCF7 cells when it was noted that NET1 expression tended to return to baseline after incubation with LPA for 4 hours (Ecimovic et al. 2014). However, the only significant change observed in CIPm was the reduction in NET1 expression. The present study does not provide enough elements for a comprehensive explanation of the phenomenon.

It is unclear how the expression of the NET1 protein is modulated by propofol. In a previous study it was suggested that propofol modulates NET1 by modifying the cellular microenvironment, rather than by a specific receptor pathway (Ecimovic et al. 2014). Other projects highlight the connection between TGF-β, microRNAs and NET1 expression as combined targets for propofol anti-metastatic activity (Shen et al. 2001; Kong et al. 2008; Lee et al.

2010; Ahmad et al. 2014), with, for instance, TGF-β increasing NET1 and mediating stress fibre formation in human keratinocytes (Shen et al. 2001).

The possible anti-cancer properties of propofol have been previously reported in several studies. For instance, propofol provoked cancer cell apoptosis in human promyelocytic leukaemia HL-60 (Tsuchiya et al. 2002) and hepatic cancer cell HepG2 (Wei et al. 2017). Propofol promoted apoptosis in pancreatic cancer cells (*i.e.* MIA-PaCa-2) in a dose-dependent manner (Du et al. 2013). In human oesophageal squamous cell carcinoma (*i.e.* EC-1) propofol inhibited *in vitro* invasion and angiogenesis and induced apoptosis through regulation of S100A4 gene expression (Guo et al. 2015). In addition, propofol suppressed the EMT and consequently kidney fibrosis through TGF-β/Smad3 signalling and regulation of miR-155 levels (Tsuchiya et al. 2002). In human MDA-MB-231 cells, propofol reduced cancer cell invasion via inhibition of nuclear factor kappa-light-chain-enhancer of activated B cell (NF-kB) pathway and subsequent reduction of matrix metalloproteinase 2 and matrix metallopeptidase 9 levels (Wei et al. 2017). Mammoto and colleagues (2002) showed that infusions of propofol at sub-anaesthetic doses for 4 weeks effectively inhibited pulmonary metastasis in mice inoculated with murine osteosarcoma cells suggesting its possible anti-invasive action *in vivo*.

Paradoxically, propofol has also been linked with mechanisms that may enhance cancer development. In gallbladder cancer cells, propofol induced proliferation and promoted invasion in a dose- and time- dependent manner through the activation of nuclear factor erythroid-derived 2 (Nrf2; from Zhang et al. 2012). In another study, human breast cancer cells (*i.e.* MDA-MB-468)

exposed to propofol showed higher migratory potential compared to controls in a dose-dependent manner (Garib et al. 2002).

6.3 Cell proliferation under sevoflurane treatments (III)

Cellular proliferation rate was effectively modified in both cell lines after treatments with a commercially available sevoflurane formulation. However, the effects on the two examined cell lines were reversed. Indeed, we observed an increase in cell proliferation rate in CIPp cells, and in CIPm cells a decrease in cell proliferation rate.

The MTT test is frequently used for the assessment of viable cell numbers. The test measures the conversion of MTT into purple formazan crystals, which are produced by the redox activity of living cells. A reduction in cellular redox activity indicates a decrement in cell viability or cell number, while an increase in cellular redox activity indicates an increment in cell viability or cell number. When studying cancer cells, an increase or decrease in cell number count can be interpreted as an increase or decrease in the cell proliferation rate (Van Meerloo et al. 2011). Therefore, in Study III it may be inferred that sevoflurane prevents the proliferation of CIPm, but enhances the proliferation of CIPp.

Our study findings are not completely in agreement with the available scientific literature. Ecimovic and co-workers (2013) demonstrated that the exposure to sevoflurane for 6 hours at concentrations of 2, 3 and 4 mM amplified cell proliferation by 50 - 63% in metastatic oestrogen receptor-positive human breast adenocarcinoma cells (MCF7 cell line) and by 50 - 67% in metastatic oestrogen receptor-negative human breast adenocarcinoma

cells (MDA-MB-231 cell line). Controversially, an anti-proliferative effect of sevoflurane at 2 mM was shown in C6 glioma cells (O'Leary et al. 2000). However, It should be considered that the C6 glioma cells derived from different tissues and were not in a tumour transformation state. Thus, possibly, underlines the role of cell type and cell evolutional phase, rather than other factors like concentration and contact time, on the ability to respond to drug exposure.

Interestingly, the divergent response of CIPp and CIPm cells proliferation rates when exposed to sevoflurane is comparable to that shown when the same cell lines were exposed to the LE propofol formulation. Indeed, propofol exposure also induced an increase and a decrease in CIPp and CIPm cell proliferation rate, respectively.

The choice of exposure times and concentrations in the present study was based on trials reported in human medicine in order to allow reasonable comparisons among studies (O'Leary et al. 2000; Ecimovic et al. 2013). However, based on sevoflurane molecular weight of 200.055 g/mol, concentrations between 1 and 4 mM correspond to concentrations of 0.2 - 0.8 mg mL^{-1}. These concentrations are actually 10 times higher than those observed in people undergoing elective cardiac surgery and receiving sevoflurane 1.8% inspiratory volume (Nitzschke et al. 2013). Considering that sevoflurane minimal alveolar concentration in dogs is only slightly higher than the one reported in humans (*i.e.* 2.10 - 2.36%; Kazama & Ikeda 1988; Scheller et al. 1990), we can assume that concentrations between 1 and 4 mM applied to cell cultures resembled plasma concentrations almost 10 times higher than those obtained in dogs anaesthetized with sevoflurane in clinical practice.

6.4 NET1 expression under sevoflurane treatments (III)

The expression of NET1 was significantly increased only in CIPm cells treated with the highest concentration used (*i.e.* 4 mM) of a commercially available sevoflurane formulation. To the authors' knowledge, this is the first study to investigate the effects of sevoflurane on the expression of NET1 gene expression. The NET1 plays a crucial role in cytoskeletal reorganization, N-cadherin expression and RhoA activation (García-Mata et al. 2007; Lee et al. 2010). Consequently, an increment in NET1 expression has been linked with more malignant cellular behaviours (Tu et al. 2010). Consistently, NET1 was demonstrated to be overexpressed in highly invasive cancer types such as human breast and gastric adenocarcinomas (Leyden et al. 2006). Expression of NET1 appears to be affected by some medications used in the perioperative period (Ecimovic et al. 2011; Ecimovic et al. 2014), as we observed also in Study II. In Study III, NET1 expression was only increased in CIPm after being exposed to the highest evaluated concentration of sevoflurane. This could be interpreted as sevoflurane enhancing the migration ability of CIPm.

6.5 Study limitations

6.5.1 *In vitro* study

The *in vitro* nature of the current studies imposes some limitations (Ghallab 2013). Initially, the mechanisms investigated are only small fragments of the wide and complex reality of cancer spread. In fact, numerous and intricate are the mechanisms that influence cancer recurrence. For instance, we cannot

exclude that propofol or sevoflurane influences cancer cells' migration and proliferation by other means such as modulation of the immune system (Yuki et al. 2010) or upregulation of hypoxia-inducible stress factors. In addition, the concomitant effects of other agents administered during routine clinical practice could possibly interfere with the effects of propofol or sevoflurane observed on cancer cells *in vitro*. For instance, serum from patients with breast cancer who received general anaesthesia with sevoflurane and systemic opioids was applied to MDA-MB-231 breast cancer cells increasing both proliferation and migration of cancer cells compared with serum of patients who received propofol infusions and paravertebral blocks (Deegan et al. 2009). Ultimately, it is difficult to extrapolate *in vitro* results to predict *in vivo* conditions. A large retrospective clinical study evaluated long-term survival of lung cancer patients undergoing volatile or intravenous anaesthesia for elective surgery. A statistically and clinically significant reduction in survival time was shown in patients receiving inhalational anaesthetics, including sevoflurane (Wigmore et al. 2016).

Since Study I did not assess the molecular characteristics of the cultured cells, the reasons for CIPp and CIPm cell lines presenting dissimilar outcomes regarding proliferation when exposed to LE propofol could not be understood.

On the same line, in the present study the expression of NET1 gene was investigated in canine mammary tumour cells without performing biological tests. Lipid-based propofol emulsion and sevoflurane clearly effectively influenced the expression of a gene closely related to increased cancer cell malignancy. However, any conclusion can be extended to the effects of LE propofol and sevoflurane on cellular behaviour. In particular, we believe it

would be beneficial to verify in future studies whether the expression of NET1 is actually correlated with increased migration potential in canine mammary tumour cells and whether the reduction in NET1 expression caused by propofol effectively decreases cell migration. Finally, it would be expected that silencing NET1 in the presence of propofol would return the migration parameters to those obtained at baseline.

6.5.2 Limitations in study design

To perform propofol dilutions, a clinically accessible propofol preparation including a lipid-based emulsion was utilized. This formulation is broadly utilized in veterinary anaesthesia and includes soybean oil, glycerol and egg lecithin as adjuvants. In a previous study in humans, the effects of an intralipid-containing propofol formulation equivalent to the one described here were compared using a MTT assay with those of a propofol-free 10% intralipid emulsion on prostate cancer cell proliferation (Huang et al. 2014). No differences were observed between the control group and the group treated with the propofol-free 10% intralipid emulsion. No reports exist that assess the blood or plasma concentrations of the lipid emulsion constituents achieved after LE propofol administration in dogs. Unfortunately, a preliminary trial to asses these parameters was not considered during the present study design. Consequently, the introduction of a sham group treated with propofol-free lipid emulsion was disregarded in the present project for two reasons. First, it was unclear to the authors what concentration of the lipid emulsion should have been used to evaluate canine tumour cell proliferation *in vitro*. Second, there was evidence in human medicine indicating no changes between intralipid-

containing propofol formulation and propofol-free 10% intralipid emulsion on cancer cell proliferation (Huang et al. 2014). However, we have to underline that the cell lines studied from Huang and colleagues were different from the ones studied in the present project. Therefore, these authors cannot fully state that the effects observed are completely due to propofol rather than to one or more of the constituents of the emulsion *per se*.

In Study I, LE propofol was diluted with cell medium to 1, 5 or 10 μg mL^{-1} while, in Study III, a clinically available sevoflurane formulation was diluted with cell medium to concentrations of 1, 2.5 or 4 mM. Dilutions of propofol and sevoflurane were performed by the same authors following instructions in papers from Ecimovic and colleagues in 2014 and 2013, respectively. However, a subsequent verification of obtained concentrations was not performed. Following these considerations, the authors cannot state to exactly know which drug concentrations were applied on cell culture.

In Study III, we utilized concentrations of Sevoflurane between 1 and 4 mM. Originally, the choice of these concentrations was done to allow comparisons with the study of Ecimovic and co-workers (2013) where sevoflurane was applied on breast cancer cells to asses proliferation, migration and invasion. These concentrations resulted to correspond to concentrations 10 times higher than those reported by Nitzschke and colleagues (2013) in people exposed to sevoflurane 1.8% inspiratory volume. Consequently, the present authors can only describe the effect of the investigated concentrations of sevoflurane. A statement regarding the effects of clinically used sevoflurane concentrations on cancer cell behaviour cannot be made.

During statistical analysis, we did not perform any test for multiple comparisons correction. With the significance level of $p < \alpha = 0.05$ chosen for the present project, it is possible to gauge from figures showing means plus minus the standard error if two conditions differ significantly by the lack of overlap between pairs of conditions. For this reason, tests for the correction for multiple comparisons were considered not necessary. However, we cannot exclude we could have observed slightly different results if a test for multiple comparisons correction would have been applied.

6.6 Practical relevance and future perspectives

In recent years, several *in vitro* studies have been focusing on the effects of anaesthetics on cancer recurrence (Melamed et al. 2003; Siddiqui et al. 2005; Deegan et al. 2009; Wada et al. 2007; Ecimovic et al. 2013; Ecimovic 2014; Song et al. 2014; Ye et al. 2014). However, the much more complex setting in the clinical practice renders challenging a direct translation of information from *in vitro* to *in vivo* scenarios. Actually, an advantage of propofol over inhalant anaesthetics when anaesthetising cancer patients for surgical cancer removal has been demonstrated by few retrospective clinical trials in human medicine (Buckley et al 2014; Wigmore et al. 2016). Conversely, data from *in vitro* trials are not entirely in agreement.

It is these authors' opinion that many are nowadays the unanswered questions regarding the relationship between cancer recurrence and anaesthetics, in particular when speaking about veterinary medicine.

Starting from the results and limits of the present study, several further investigations can be suggested. The role of intralipid emulsion on canine cancer cell should be examined to better understand whether changes in cell behaviour when applying intralipid-containing propofol formulations are due to propofol molecules *per se* or not. Different cell lines could be utilized to analyse canine cancer cell response to propofol and sevoflurane, since we cannot exclude different reactions between different cell lines. More research should be performed on other aspects of cancer cell behaviour such as invasion, migration and the expression of oncogene others than NET1. Finally, it would be interesting to study a wider range of concentrations of propofol and sevoflurane compared with those here reported.

During the time spent in reading about the topic of this dissertation, the authors found fascinating the amount of information available in human medicine regarding the possible effects of drugs others than propofol and sevoflurane on cancer recurrence. Indeed, we would consider beneficial a deep investigation on the role of opioids and local anaesthetics on cancer recurrence.

Not only for the potential benefits of canine patients, but also due to recent recognition of canine mammary tumours as possible translational models of naturally occurring breast cancer in people (Alvarez 2014), the authors advocate clinical veterinary trials on canine patients affected with mammary tumours to achieve a wider understanding of the influence of anaesthetic techniques on cancer recurrence. Ideally, we would suggest the development of prospective randomised blinded clinical trials involving canine females affected with mammary cancer. The aim would be to compare the metastasis-

free time after surgery between canine patients anaesthetized with propofol or sevoflurane and receiving an opioid-based or a local anaesthetic-based analgesia. The goal would be to identify which anaesthetic protocol results in the longer metastasis-free time after surgery.

7. CONCLUSIONS

1. Exposure to LE propofol significantly influenced canine mammary tumour cell proliferation rate. Higher but still clinically relevant concentrations of LE propofol and longer exposure times resulted in a significant increase in cell proliferation when applied to primary canine mammary tumour cells and a significant decrease in cell proliferation when applied to metastatic canine mammary tumour cells (Study I).
2. Exposure to LE propofol significantly influenced canine mammary tumour cell NET1 expression. Both examined concentrations induced a significant decrease in gene expression at most of the treatment time points, although increases in gene expression were also observed (Study II).
3. Exposure to sevoflurane significantly influenced canine mammary tumour cell proliferation rate. Significant increases or decreases of cell proliferation rate were observed when sevoflurane was applied to primary or metastatic canine mammary tumour cells, respectively, compared with cells grown solely in the cell culture medium (Study III).
4. Exposure to sevoflurane significantly influenced canine mammary tumour cell NET1 expression, but only when the higher concentration of sevoflurane was applied to the metastatic canine mammary tumour cells (Study III).

Further studies, including biological tests, gene silencing and clinical trials, are warranted to elucidate the role of anaesthetic techniques in cancer recurrence.

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